



TITLE:

# 原始被子植物の分子進化と生殖・繁殖機構の研究

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# 原始被子植物の分子進化と生殖・繁殖機構の研究

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15. 岡田潤・徳岡徹・東浩司・戸部博：広義ヤナギ科の分子系統解析。日本植物学会第 68 回大会（藤沢）9 月 9 日－12 日。
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2. 十河暁子・戸部博：ヤマモモ（ヤマモモ科）の花粉管伸長とブナ目における珠孔を使わない受精様式の進化。日本植物分類学会第 4 回大会（高知）3 月 11 日－13 日。
3. 門川朋樹・戸部博：ショウブ属の生殖器官の発生学的研究：特に胚珠の構造について。日本植物分類学会第 4 回大会（高知）3 月 11 日－13 日。
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**The evolution of fertilization modes independent of the micropyle in  
Fagales and 'pseudoporogamy'**

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Running title: Evolution of fertilization modes in Fagales

**Abstract.** In contrast to a majority of angiosperms that show porogamous fertilization, several fagalean families such as Betulaceae and Casuarinaceae are known to show chalazogamy, where fertilization is effected by a pollen tube passing through the chalaza instead of the micropyle. Our developmental study of pollen-tube growth in pistils of Myrica rubra (Myricaceae, Fagales) further shows that pollen tubes reached the nucellus before the micropyle is formed by the integument. Since fertilized ovules appeared as if the pollen tube had passed through the micropyle for fertilization, we propose the new term 'pseudoporogamy' to this mode. By mapping diverse modes of fertilization, dependent or independent of the micropyle, onto a phylogenetic tree of Fagales, it appears that fertilization mode evolved from porogamy to chalazogamy and then further from chalazogamy to pseudoporogamy. Possible reasons for the evolution of fertilization modes independent of the micropyle in Fagales are discussed.

**Key words:** Fagales, fertilization, micropyle, Myrica rubra, Myricaceae, pollen-tube growth

## Introduction

In a majority of angiosperms, a pollen tube enters the ovule for fertilization through the so-called micropyle formed by the integument(s). This mode of fertilization in relation to pollen-tube path is known as 'porogamy' (Maheshwari 1950). In Fagales (with eight families), however, while porogamy is known from Fagaceae (e.g., Benson 1894; Hjelmquist 1953), chalazogamy, where the pollen tube enters the ovule for fertilization through the chalaza instead of the micropyle, is also known from Betulaceae and Casuarinaceae (Sogo et al. 2004a, b; Sogo and Tobe 2005; references cited therein).

In a series of developmental studies on pollen-tube growth mode in pistils of Fagales, we have noticed that in *Myrica rubra* Siebold & Zucc. (Myricaceae), which has a single unitegmic and orthotropous ovule per ovary, pollen tubes reach the nucellus before the micropyle is formed; in other words, they enter the ovule without using the micropyle (Sogo and Tobe 2006), reminding us of the aforementioned chalazogamy in Betulaceae and Casuarinaceae. This suggests that several different modes of fertilization in relation to pollen-tube path are likely to have evolved in Fagales, and in this paper we describe a distinct mode of fertilization in *Myrica rubra* in terms of pollen-tube path. Based on analyses of diverse modes of fertilization in Fagales, we will examine evolutionary trends of fertilization mode in the order and why fertilization modes independent of the micropyle have evolved.



## Materials and methods

Fertilization and pollen-tube growth in pistils of *Myrica rubra* were examined under the light microscope using microtome sections of a total of 115 pistils in various developmental stages from the time of pollination to the time of (or just after) fertilization. Scanning electron micrographs (SEMs) of over 20 ovules before and after fertilization were also examined. To obtain those pistils or ovules, female inflorescences from trees cultivated along the roadside in the city of Kyoto, Japan, were collected every three or four days from mid-April to mid-May in 2004. They were fixed in FAA (5% stock formalin; 5% glacial acetic acid; 90% ethanol (50%)). Methods to prepare microtome sections and SEMs were described elsewhere (Sogo and Tobe 2006). Although we could not anatomically examine exact developmental stages of ovules that were used for SEMs, we estimated them from microtome sections of ovules that were collected from the same inflorescence and had similar external ovule morphology.

In this paper we will focus on the formation of the micropyle. We apply the term micropyle strictly when an apical opening of the integument is tightly closed in microtome sections and SEMs. If it still looks open, we score it as micropyle not formed yet.

We mapped diverse fertilization modes on a phylogenetic tree of Fagales using MacClade version 3.04 (Maddison and Maddison 1992). The phylogenetic tree was

adopted from Li et al. (2004). All the character-states were unordered, unpolarized and unweighted, and missing values were scored as '?'.

## Results and discussion

Each female inflorescence has 10-25 pistillate flowers, and the pistillate flower has a small unilocular ovary with two elongate stigmas from the time of pollination up to one week after pollination (Fig. 1A). In the ovary a single ovule develops from the basal placenta, which is unitegmic and orthotropous with the nucellus apex facing upward (Fig. 1B). When the stigmas receive many pollen grains, the ovary (or ovule) is still immature, or more exactly at the megaspore mother cell stage, so that fertilization is delayed for between two and three weeks. Although more than 20 pollen tubes per stigma germinated and grew into the tissue of the stigma, they stay within the ovary for over two weeks until the ovule becomes mature enough for fertilization (Sogo and Tobe 2006). Prior to reaching a mature embryo sac for fertilization, the pollen tubes stay in the upper space of the ovary locule and on the surface of the nucellus, according to the developmental stages of the ovary (or ovule). As reported Sogo and Tobe (2006), one to three pollen tubes (more exactly, tips of branched pollen tubes) reached from the upper space of the ovarian locule to the surface of the nucellus when, or soon after, the ovule reached the megaspore tetrad stage (Fig. 1C, D, E). The integument apex is still widely open; in other words, the 'micropyle' is not yet formed (Fig. 1C, E), developing in later stages.

Observations of 115 ovules (or seeds) in different developmental stages showed

when the micropyle was formed, with or without relation to the positions of the pollen tubes (Table 1). Although all the ovules possess a micropyle after fertilization, not all of them had formed it before fertilization or even at the mature embryo sac stage, and of 21 ovules just before or after fertilization, only 10 had formed the micropyle. Examination of 17 ovules at the four-nucleate embryo sac stage revealed that half of them (nine ovules) lacked the micropyle (Fig. 1F, G). This was also the case for ovules at the two-nucleate embryo sac stage. As no ovules at the dyad megaspores stage or younger ovules possessed a developed micropyle, if it is present at the stage of fertilization, then appears to be formed some time between the two-nucleate and mature embryo sac stages. Nevertheless, pollen tubes had already reached the nucellar surface in all the ovules at the megaspore tetrad stage, that is, before micropyle formation. Earlier studies of *Myrica rubra* all reported that fertilization was porogamous (Treub 1891; Yen 1950; Håkansson 1955). This must be corrected because the pollen tube actually reaches the nucellus before the micropyle is formed.

However, in fertilized ovules, it looks as if the pollen tube had passed through the developed micropyle and appears to be porogamous (Fig. 1H, I). The authors, who described that fertilization was porogamous in *Myrica rubra* (Treub 1891; Yen 1950; Håkansson 1955), may have been deceived by the appearance of the fertilized ovules. As we reported in Sogo and Tobe (2006), of one to three pollen tubes that reached the



nucellar surface at the megaspore tetrad stage and then adhered there with their thickened tips for two weeks, only one regerminated and entered the nucellus to reach a mature embryo sac for fertilization (Fig. 2). Accordingly, we call this mode of fertilization 'pseudoporogamy' in which the pollen tubes reach the nucellus or the embryo sac prior to micropyle development.

As stated in the Introduction, chalazogamous pollen tubes do not use the micropyle but reach an embryo sac through the chalaza. Chalazogamy has been reported from Betulaceae (Nawaschin 1893; Benson 1894; Nawaschin 1895, 1899a, 1899b; Benson et al. 1906; Wolpert 1910; Finn 1936), Casuarinaceae (Treub 1891; Frye 1903; Swamy 1948; Barlow 1958; Sogo et al. 2004a, b), and Juglandaceae pro parte (Karsten 1902; Billings 1903; Langdon 1934; Nast 1935, 1941; Sartorius and Anvari 1984; Luza and Polito 1991). In contrast, porogamy has been reported from members of the Fagaceae (Benson 1894; Hjelmquist 1953; Boavida et al. 1999; Nakamura 2001) which are phylogenetically basal in Fagales (Li et al. 2004).

Various modes of fertilization were mapped with data of outgroups *Hamamelis* and *Celtis* taken from Mathew (1980) and Dottori (1994) onto a phylogenetic tree of Fagales derived from Li et al. (2004) (Fig. 3). It shows that evolution from porogamy to chalazogamy occurred once in the common ancestor of Juglandaceae, Rhoipteleaceae, Myricaceae, Betulaceae, Ticodendraceae and Casuarinaceae (although the fertilization

mode is not known yet in Rhoipteleaceae and Ticodendraceae) and that evolution from chalazogamy to pseudoporogamy subsequently occurred in the common ancestor of Myricaceae. Pseudoporogamy currently appears to be restricted to Myricaceae. However, it may also occur in Rhoipteleaceae (Rhoiptelea only) and uninvestigated taxa of Juglandaceae. In Juglans both chalazogamy and porogamy have been reported (Nast 1935; Schanderl 1964; Luza and Polito 1991). However, 'porogamy' in Juglans needs reconfirmation, since it may be pseudoporogamy as seen in Myrica.

It is of interest why the modes of fertilization independent of the micropyle, i.e., chalazogamy and pseudoporogamy, have evolved in Fagales. In Casuarina equisetifolia (and probably all of the rest of the Casuarinaceae), which have hemitropous ovules similar to anatropous ones, as in the basal Fagales (Fagaceae and Nothofagaceae), delayed fertilization (see Endress 1977, Fig. 10) provides a sufficient time for (male and female) gametophyte selection (Sogo et al. 2004a). For some time before fertilization, the pollen tubes stay on the funiculus and in the chalaza (Sogo et al. 2004a). The thick tissue of the funiculus and chalaza, which are supplied by a massive vascular bundle, may provide nutrition for the pollen tube that has to survive in the pistil until the embryo sac matures. If chalazogamy was once established in the common lineage of Casuarinaceae, Ticodendraceae, Betulaceae, Myricaceae, Rhoipteleaceae and Juglandaceae, the pollen tube may no longer need the micropyle as the path to reach the embryo sac in the

orthotropous unitegmic ovule that has evolved in Myricaceae and Juglandaceae. In Myrica the apical surface of the nucellus, instead of the chalaza, functions as a source of nutrition for pollen tubes and cells of the nucellar apex are rich in starch grains in M. rubra, suggesting that the pollen tubes may digest them while adhering to the nucellar surface (Sogo and Tobe 2006). Both chalazogamy and pseudoporogamy may have evolved in response to the requirement of a nutrition source by the pollen tubes staying in a pistil for a long period before fertilization after pollination.

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## FIGURE LEGEND

**Fig. 1.** Ovule development in *Myrica rubra*. **B, C, D, F, H** microtome sections; **E, G, I** scanning electron micrographs (SEM). **A** a pistil about one week after pollination; the stigmas started withering from their tips; **B** longitudinal section (LS) of an ovary with a single orthotropous ovule; **C** part of **B**, magnified to show that pollen tubes (arrows) reach the nucellar surface before the tip of integument closes to form the micropyle; **D** part of **C** (rectangle), magnified to show a tetrad of megaspores (arrowheads); **E** SEM showing an apical part of an ovule, estimated at the megaspore tetrad stage; tips of branched pollen tubes (arrows) enter the ovule through the apical opening of the integument; **F** LS of ovule at the four-nucleate embryo sac stage; **G** SEM showing an apical part of an ovule, estimated at the 4-nucleate embryo sac stage; arrows indicate pollen tubes; **H** LS of ovule (seed), estimated at the stage of free endosperm nuclei; **I** SEM showing an apical part of ovule (seed) with free endosperm nuclei; the micropyle is now formed by closing of the tip of the integument; arrows indicate pollen tubes. Abbreviations: em embryo; en endosperm nucleus; i integument; n nucleus in the embryo sac; ov ovule; ova ovary; sg stigma; sy style. Scales: **A** = 1 mm; **B** = 200 $\mu$ m; **C, E, G, H** = 50 $\mu$ m; **D, F** = 20 $\mu$ m.

**Fig. 2.** Diagrammatic representation of 'pseudoporogamy' in *Myrica rubra*. **A** ovule at the megaspore tetrad stage; **B** ovule at the 4-nucleate embryo sac stage; **C** ovule after



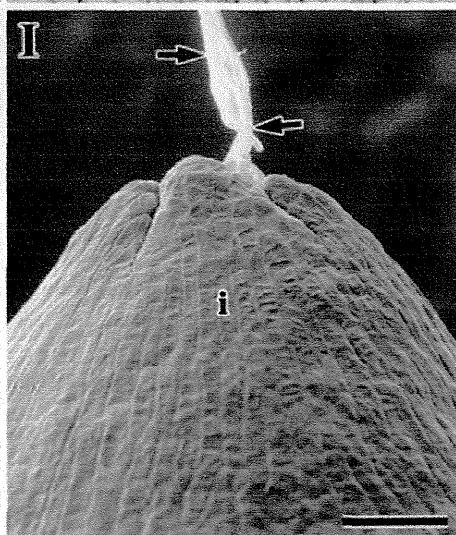
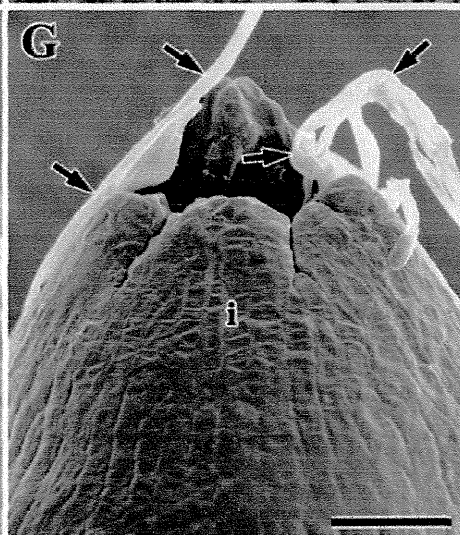
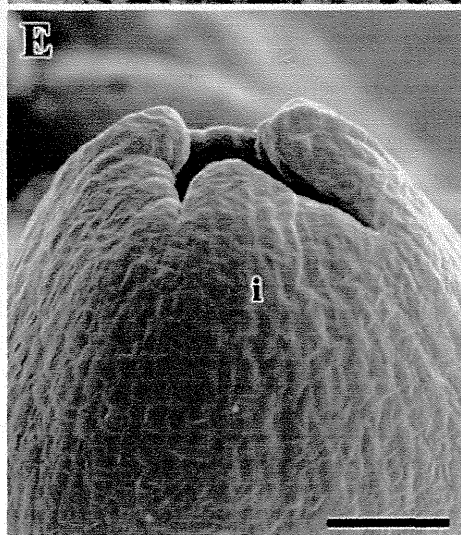
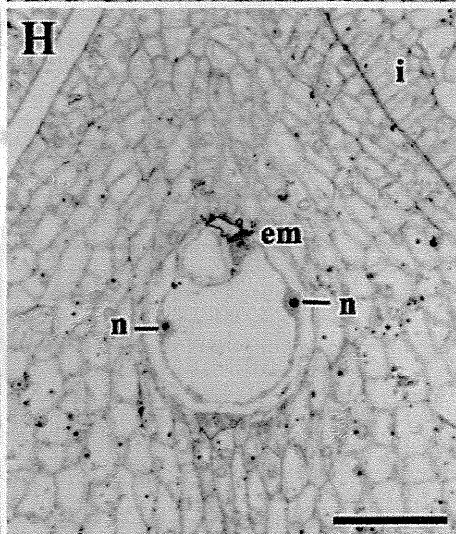
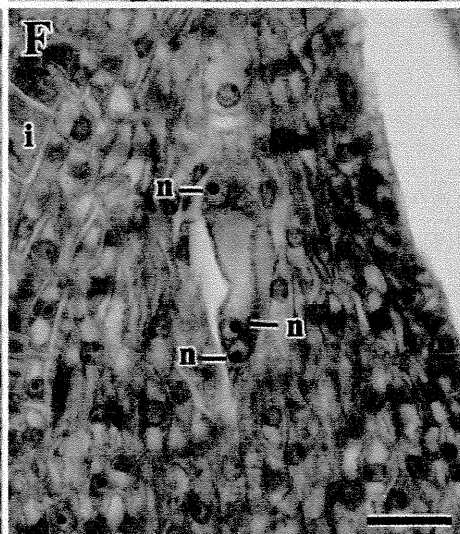
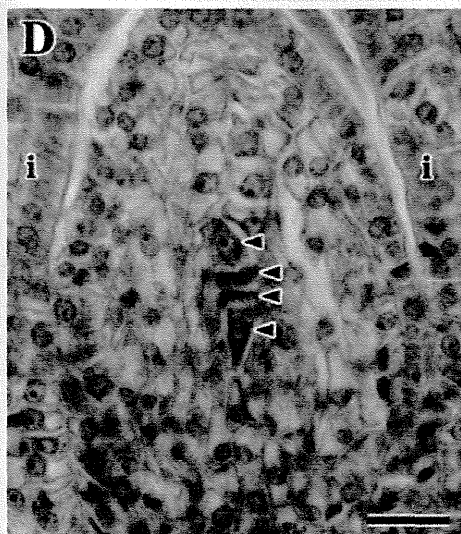
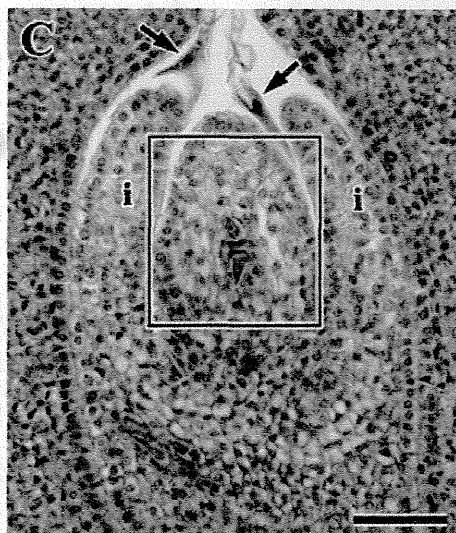
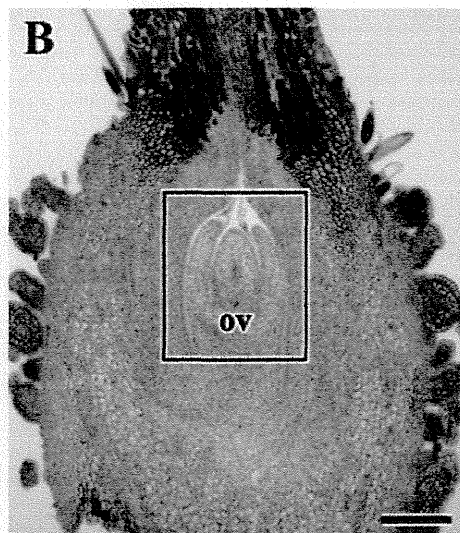
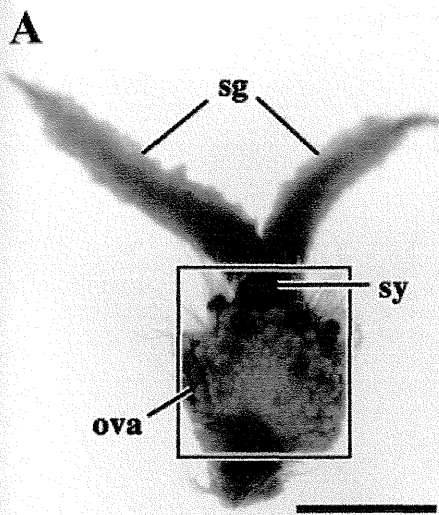
fertilization; pollen tubes reached the nucellar surface before the micropyle was formed, so that a fertilized ovule looks as if fertilization was effected by a pollen tube passing through the micropyle. Abbreviations: em embryo; es embryo sac; i integument; pt pollen tube.

**Fig. 3.** Phylogenetic tree of Fagales redrawn from Li et al. (2004), showing an evolutionary trend of modes of fertilization from porogamy to chalazogamy and further to 'pseudoporogamy.'

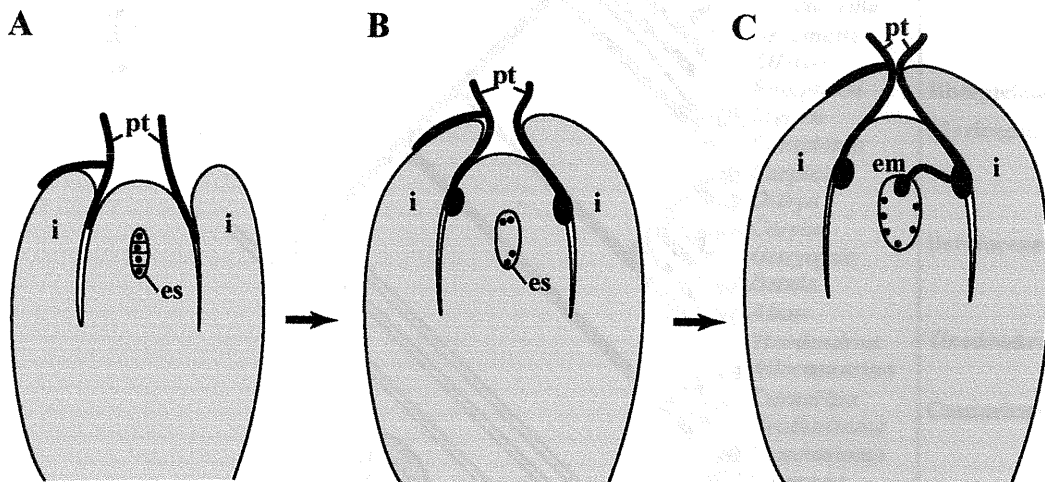
Table 1. Positions of pollen tubes in the pistil in association with the development of ovules, and formation of the micropyle in *Myrica rubra*. Figures indicate the number of ovules observed.

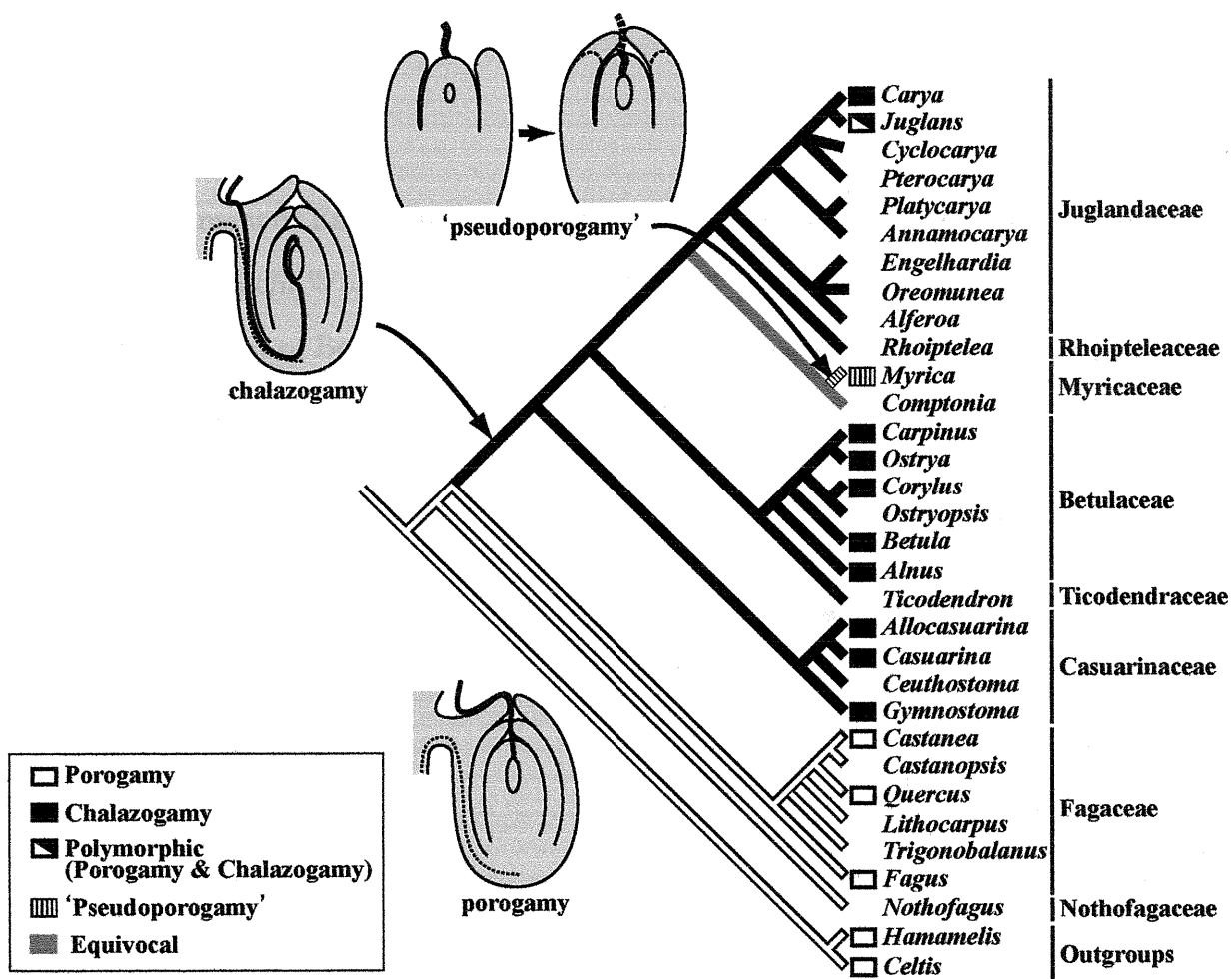
	Developmental stages of ovules							After fertilization
	Megaspore mother cell stage	Megaspore dyad stage	Megaspore tetrad stage	1-nucleate embryo sac stage	2-nucleate embryo sac stage	4-nucleate embryo sac stage	Mature embryo sac stage	
Position of pollen-tube tip(s)	Ovarian locule		Surface of the nucellus				Embryo sac	—
Micropyle								
Not formed	34	3	17	0	4	9	11*	0
Formed	0	0	0	0	4	8	10*	15

\*including ovules with the embryo sac just after fertilization.









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**Chromosome evolution in the Laurales based on analyses of  
original and published data**

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## Abstract

We present a summary of currently available chromosome information for all seven families in the order Laurales on the basis of original and previously published data and discuss the evolution of chromosomes in this order. Based on a total of 53 genera for which chromosome data were available, basic chromosome numbers appear consistent within families:  $x = 11$  (Calycanthaceae);  $x = 22$  (Atherospermataceae and Siparunaceae);  $x = 19$  (Monimiaceae); and  $x = 12$  and  $15$  (Lauraceae). The Hernandiaceae have diverse numbers:  $x = 15$  (Gyrocarpoideae) and  $x = 10$  and  $x = 18$  (Hernandioideae). Karyotype analyses showed that *Hennecartia*, *Kibaropsis*, and *Matthaea* (all Monimiaceae) contained two or three sets of four distinct chromosomes in 38 somatic chromosomes, suggesting that  $2n = 38$  was derived by aneuploid reduction from  $2n = 40$ , a tetraploid of  $x = 10$ . In light of the overall framework of phylogenetic relationships in the Laurales, we show that  $x = 11$  is an archaic base number in the order and is retained in the Calycanthaceae, which are sister to the remainder of the order. Polyploidization appears to have occurred from  $x = 11$  to  $x = 22$  in a common clade of the Siparunaceae, Atherospermataceae, and Gomortegaceae (although  $2n = 42$  in the Gomortegaceae), and aneuploid reduction from  $x = 11$  to  $x = 10$  occurred in a common clade of the Hernandiaceae, Lauraceae, and Monimiaceae. To understand chromosome evolution in the Lauraceae, however, more studies are needed of genera and species of Cryptocaryae.

**Key words** Chromosome • Evolution • Hernandiaceae • Lauraceae • Laurales • Monimiaceae

## Introduction

The order Laurales, which comprises seven families and 93–94 genera that are distributed worldwide in the tropics and subtropics (Renner 1999; Stevens 2005), is a member of the basal angiosperms, the magnoliids (The Angiosperm Phylogeny Group [APGII] 2003). Recent phylogenetic analyses have shown that in the Laurales, the Calycanthaceae are the first-branching group, followed by the Atherospermataceae–Gomortegaceae–Siparunaceae clade and the Hernandiaceae–Lauraceae–Monimiaceae clade (Renner 1999; Qiu et al. 2005; Stevens 2005). In the former clade, the Siparunaceae are basal and sister to the Atherospermataceae–Gomortegaceae subclade, while in the latter clade, the Monimiaceae are sister to the Hernandiaceae–Lauraceae subclade. Relationships within individual families such as the Lauraceae (Rohwer 2000; Chanderbali et al. 2001), Hernandiaceae (Renner and Chanderbali 2000), and Monimiaceae (Renner 1998) are also understood relatively well. Using an overall framework of such phylogenetic relationships in the Laurales as well as in individual families, we can attempt not only to trace evolutionary trends of various morphological characters in this order but also to predict missing data. Renner (1999) discussed the evolution of apical versus basal ovule placentation, the presence or absence of nectary glands in a flower, and the anther dehiscence mode by slits or valves. The second author and his colleagues have published a series of embryological studies of different families (Siparunaceae [Kimoto and Tobe 2003]; Gomortegaceae [Heo et al. 2004]; Lauraceae [Kimoto et al. 2006]) and have discussed their evolution in the Laurales. While investigating chromosome data of *Eusideroxylon* (Heo and Oginuma 1994) and *Endiandra* (Oginuma et al. 1999) in the Lauraceae, we collected additional data on the other families in the Laurales, in particular the Hernandiaceae and Monimiaceae, to fill information gaps whenever materials were available.

Ehrendorfer et al. (1968) summarized chromosome data available for the



Laurales in their review paper of chromosome numbers of "primitive" angiosperms. They suggested the base number  $x = 11$  for the Calycanthaceae,  $x = 22$  for the Siparunaceae,  $x = 19$  for several southwestern Pacific genera of Monimiaceae, and  $x = 12$  for the Lauraceae, and even discussed the possible origin of a part of those base numbers. Okada and Tanaka (1975) confirmed the base number  $x = 12$  for the Lauraceae on the basis of their original data of 16 species in nine genera. However, because very little chromosome information and no reliable information on phylogenetic relationships were available at that time, they could not provide a straightforward discussion on chromosome evolution.

To date, chromosome data have been published for 51 of the 93-94 genera in the seven families of Laurales, although nearly all earlier studies reported chromosome numbers alone without analyses of karyotypes of somatic chromosomes at metaphase. In this paper, except one case in which too many chromosomes were observed to be analyzed, we report the karyotypes of seven genera and eight species in the Hernandiaceae, Lauraceae, and Monimiaceae, for which chromosome information is still sparse. Combining our original data with previously published data, we discuss the evolution of chromosomes in the Laurales and suggest additional research topics to further our understanding of chromosome evolution in this order.

### Materials and methods

The species of Hernandiaceae, Lauraceae, and Monimiaceae that we investigated to obtain original chromosome data are presented in Table 1 along with their collection data and chromosome numbers. Somatic chromosomes were examined using either root tips collected from seedlings (*Illigera trifoliata* Dunn, *Cryptocarya laevigata* Blume, *Hennecartia omphalandra* Poisson, and *Kibaropsis caledonica* [Guillaumin] Jérémie) or young leaves collected from trees growing in the field

(Hernandia bivalis Benth., H. ovigera L., Kibara macrophylla [R. Cunn.] Benth., Matthaea calophylla Perkins, and Sparattanthelium amazonum Pilg.). Methods of pretreatment, fixation, and staining for chromosome observations followed Oginuma and Nakata (1988) and Oginuma et al. (1992). At least three to five cells were examined to determine the karyotype of each species.

Terminology of chromosome morphology on the basis of the position of centromeres followed Levan et al. (1964). For comparison among different karyotypes at mitotic metaphase, we used the karyotype formula of Kim et al. (2003, p. 49) and more recently Meng et al. (2005). For example, in Hernandia bivalis, where  $2n = 40 = 32m + 4sm + 4st$ , 40 somatic chromosomes are composed of 32, four, and four chromosomes that have a centromere at the median (m), submedian (sm), and subterminal (st) positions, respectively. In addition, in a case in which several chromosomes have a secondary constriction (SC), it is expressed as  $m^{SC}$  or  $sm^{SC}$ .

For critical comparisons, we presented data on size measurements of 38 individual somatic chromosomes in Hennecartia omphalandra, Kibaropsis caledonica, and Matthaea calophylla in Tables 3–5. The 38 chromosomes were numbered from long to short ones according to their length. Chromosome “lengths” are indicated in the order: short arm + long arm = total length, and in cases in which the long arm is divided by a SC into two elements, their respective lengths are presented. “Relative length” means the percentage of the length of each chromosome to the total length of 38 chromosomes. The “arm ratio” is a value obtained by dividing the length of a long arm by the length of a short arm. The “shapes” (m,  $m^{SC}$ , sm,  $sm^{SC}$ , st) in the right column are as above.

We examined all published data on the Laurales using Chromosome Numbers of Flowering Plants by Fedorov (1974), serial publications, and books titled Index to Plant Chromosome Numbers (Ornduff 1967–1968; Moore 1973–1977; Goldblatt 1981–1988; Goldblatt and Johnson 1990–2003). When we encountered

questionable counts that often appeared in older literature, we checked them against original articles.

## Results and discussion

### Calycanthaceae

The Calycanthaceae comprise five genera and 11 species, and  $2n = 22$  and  $33$  are reported for seven species in the four genera Calycanthus, Chimonanthus, Idiospermum, and Sinocalycanthus (Table 2). The base number of each genus as well as of the whole family is  $x = 11$ .

### Atherospermataceae-Gomortegaceae-Siparunaceae clade

The Atherospermataceae comprise six or seven genera and 16 species. The chromosome number is reported from one species each of the four genera Atherosperma, Daphnandra, Doryphora, and Laurelia. Among these, both Daphnandra and Laurelia have  $2n = 44$ , whereas Atherosperma has  $n = 21$ . Doryphora is reported to have  $n = \text{ca. } 40$  or  $2n = \text{ca. } 82$ , but these numbers require reconfirmation (Table 2). Together, all available data suggest that Daphnandra and Laurelia have  $x = 22$ , and  $2n = 44$  is likely to be a tetraploid of  $x = 11$ .

The family Gomortegaceae comprises only Gomortega nitida Ruiz & Pavon (syn. G. keule). In the only article available on the chromosome number for this family, Goldblatt (1976) reported  $2n = 42$  (Table 2).

The Siparunaceae comprise two genera, Glossocalyx (G. longicuspis Benth. only) and Siparuna (74 species). Chromosome data are known only from three species of Siparuna, for which  $n = 22$  and  $44$  and/or  $2n = 44$  and  $88$  are reported (Table 2). The base number of the genus as well as of the family is  $x = 22$ , and  $2n = 44$  is likely a tetraploid with  $x = 11$ .



### Hernandiaceae-Lauraceae-Monimiaceae clade

The Hernandiaceae, which comprise 57 species in five genera, are divided into the two subfamilies Gyrocarpoideae (Gyrocarpus and Sparattanthelium) and Hernandioideae (Hazomalania, Hernandia, and Illigera; Buchheim 1964; Kubitzki 1993). Such a subfamilial division is supported by molecular evidence (Renner and Chanderbali 2000). In the Hernandioideae, Hernandia is sister to the Hazomalania-Illigera clade (Renner, pers. comm.). Of these five genera, two (Gyrocarpus and Hernandia) have been studied cytologically (Table 2). Here we add chromosome data for two additional species (H. bivalis and H. ovigera) of Hernandia as well as of Illigera and Sparattanthelium.

Both Hernandia bivalis and H. ovigera, which were investigated here for the first time, have  $2n = 40$ . Chromosomes at metaphase gradually varied in length from about 2.5  $\mu\text{m}$  to about 0.8  $\mu\text{m}$  in H. bivalis (Figs. 1, 2) and from 3.7  $\mu\text{m}$  to 1.0  $\mu\text{m}$  in H. ovigera (Figs. 3, 4). In the two species, 32 of 40 chromosomes had a centromere at the median position; of the eight remaining chromosomes, four each had a centromere at the submedian and subterminal positions, respectively. No satellite chromosomes were observed. Their karyotype formula is  $2n = 40 = 32m + 4sm + 4st$ . The 40 chromosomes are composed of four sets of ten chromosomes, each comprising  $8m + 1sm + 1st$ . Earlier records of chromosome numbers for Hernandia are  $n = 20$  and  $40$  and  $2n = 40$  (Table 2). Taken together, all available chromosome data show that the base number of Hernandia is  $x = 20$ , and that  $2n = 40$  is a tetraploid with  $x = 10$ .

Illigera trifoliata, which represents the first investigation of this genus, had  $2n = 36$  (Figs. 5, 6). Chromosomes at metaphase gradually varied from 2.1  $\mu\text{m}$  to 1.2  $\mu\text{m}$ . Of the 36 chromosomes, 30 had a centromere at the median position; of the six remaining chromosomes, two and four had a centromere at the submedian and subterminal positions, respectively. A SC was observed in the proximal region of a

short arm in two of the 30 metacentric chromosomes. The karyotype formula is  $2n = 36 = 28m + 2m^{SC} + 2sm + 4st$ . The base number of the genus is  $x = 18$ , but more species must be studied to confirm this number.

Sparattanthelium amazonum had  $2n = 30$  (Figs. 7, 8) as in Gyrocarpus (with  $n = 15$  and  $2n = 30$ ). Morawetz (1986) reported  $2n = ca. 48$  in S. cf. amazonum and  $2n = ca. 96$  in S. botocudorum Mart. Since the quality of the photograph (Fig. 10b, p. 64 in Morawetz 1986) is of poor quality, these numbers need reconfirmation. Thirty chromosomes in our materials gradually varied from about  $2.2 \mu m$  to  $0.9 \mu m$ . Of the 30 chromosomes, 22, two, and six had a centromere at the median, submedian, and subterminal positions, respectively. Satellite chromosomes were not observed. Thus the karyotype formula is  $2n = 30 = 22m + 2sm + 6st$ . The base number of both Gyrocarpus and Sparattanthelium is  $x = 15$ .

In summary, the Hernandiaceae have diverse basic chromosome numbers, i.e.,  $x = 15$  in Gyrocarpus and Sparattanthelium (Gyrocarpodeae),  $x = 18$  in Illigera, and  $x = 20$  in Hernandia.

The Lauraceae are the largest family in the Laurales, comprising about 50 genera and 2500 species. With regard to their phylogenetic relationships, the Cryptocaryeae (Aspidostemon, Beilschmiedia, Caryodaphnopsis, Cassytha, Cryptocarya, Endiandra, Eusideroxylon, Hypodaphnis, Potameia, and Potoxylon) are the first-branching lineage, successively followed by the Chlorocardium-Mezilaurus clade, the Persea group, Laureae, and Cinnamomeae (Chanderbali et al. 2001; Kimoto et al. 2006). Of the 50 genera, 23 have been studied cytologically (Table 2). Most of the genera have  $2n = 24, 36$ , or  $48$ ; therefore, the base number is  $x = 12$ . Exceptionally, however, both Eusideroxylon (Heo and Oginuma 1994) and Endiandra (E. brasii C. K. Allen [Cryptocaryeae]; Oginuma et al. 1999) have  $2n = 30$ . We examined chromosomes of Cryptocarya laevigata (Cryptocaryeae) with the expectation that we would find  $2n = 30$  in more species of

Cryptocaryae. However, this species had  $2n = 24$  (Figs. 9, 10) in agreement with the earlier records, i.e.,  $n = 12$  in Cryptocarya amygdalina Nees and C. floribanda Nees (Mehra and Bawa 1969). Therefore, the base number of Cryptocarya is  $x = 12$ . In the case of C. laevigata, 24 chromosomes were gradual in length from about 2.2  $\mu\text{m}$  to about 0.9  $\mu\text{m}$ . Twenty-two of 24 chromosomes had a centromere at the median position, and the remaining two had a centromere at the submedian position. Satellite chromosomes were not observed. The karyotype formula is  $2n = 24 = 22m + 2sm$ .

The Monimiaceae are the second largest family, comprising 24 genera and 270 species. This family is divided into three subfamilies: Monimioideae (Monimia, Palmeria, and Peumus), Hortonioideae (Hortonia), and Mollinedioideae (20 remaining genera; Philipson 1993). The Monimioideae are the first-branching group, followed by the Hortonioideae and Mollinedioideae (Renner, pers. comm.). Among the 24 genera, 16 have been examined cytologically, but several of these, including Ehippiandra, Hennecartia, Kibara, Monimia, and Peumus, have vague or dubious counts. For example, Morawetz (1981) reported  $2n = 78$  in Peumus (which includes only P. boldus), but we counted 76–78 chromosomes in the same figure (Fig. 2a on p. 162 in Morawetz 1981) that show chromosomes at prometaphase. The exact chromosome numbers therefore need reconfirmation by considering only chromosomes that are strictly at metaphase. In addition, karyotype analyses are required on any species to consider possible evolutionary links to other families. We added chromosome data from the four genera Hennecartia, Kibara, Kibaropsis, and Matthaea. Kibaropsis (K. caledonica only) was already reported to have  $n = 19$ , but no data were available on somatic chromosomes; no chromosome data were available for Matthaea.

Hennecartia omphalandra had  $2n = 38$  (Figs. 11, 12). The previous count of  $2n = \text{ca. } 96$  (Morawetz 1986) seems erroneous. Their length varied gradually from 1.8



$\mu\text{m}$  to  $0.6 \mu\text{m}$  (Table 3). Of the 38 chromosomes, 30, four, and four had a centromere at the median, submedian, and subterminal positions, respectively. Four acrocentric chromosomes (i.e., chromosomes with a centromere at the subterminal position) were longer than other chromosomes (see white arrows in Figs. 11, 12), and four submetacentric chromosomes were subsequently longer than the other chromosomes (Table 3). The SC was observed in the proximal region of a short arm in the four metacentric chromosomes. The karyotype formula is  $2n = 38 = 26m + 4m^{SC} + 4sm + 4st$ . The base number of the genus is thus  $x = 19$ .

Kibara macrophylla, which was investigated here for the first time, had  $2n = 114$  (Figs. 13, 14). Their length varied gradually from about  $1.5 \mu\text{m}$  to  $0.6 \mu\text{m}$ . Of the 114 chromosomes, while many chromosomes were unclear with respect to the position of a centromere, several had a centromere at the median, submedian, or subterminal positions. Satellite chromosomes were not observed. The species is a hexaploid with  $x = 19$ .

Kibaropsis caledonica had  $2n = 38$  (Figs. 15, 16). The chromosomes showed a bimodal variation in chromosome length; of the 38 chromosomes, two were longer and about  $2.5\text{--}2.1 \mu\text{m}$  long, and 36 were shorter and about  $1.6\text{--}1.0 \mu\text{m}$  long (Table 4). Irrespective of their length, 26, six, and six chromosomes had a centromere at the median, submedian, and subterminal positions, respectively. Of the six acrocentric chromosomes, two were longer than all other chromosomes, and four were shorter (see white arrows in Figs. 15, 16, Table 4). The SC was observed in the interstitial region of a long arm in four of the six submetacentric chromosomes. The karyotype formula is  $2n = 38 = 26m + 2sm + 4sm^{SC} + 6st$ . Taken together with the previous report of  $n = 19$ , our data show that the base number of this genus is  $x = 19$ .

Matthaea calophylla, which represented the first investigation of this genus, had  $2n = 38$  (Figs. 17, 18). Their length gradually varied from  $2.5 \mu\text{m}$  to  $0.6 \mu\text{m}$  (Table 5). Of 38 chromosomes, 26, eight, and four had a centromere at the median,

submedian, and subterminal positions, respectively. Of the eight submetacentric chromosomes, four that were longest of all chromosomes had a SC at the interstitial region of a long arm, and the other four were subsequently longer than the other chromosomes (Table 5). The four acrocentric chromosomes were shorter of all chromosomes (Table 5). The karyotype formula is  $2n = 38 = 26m + 4sm + 4sm^{SC} + 4st$ . The base number of this genus is  $x = 19$ .

In summary, of the 24 genera in the Monimiaceae, nine (Hedycarya, Hennecartia, Hortonia, Kibara, Kibaropsis, Levieria, Matthaea, Palmeria, and Tambourissa) have  $x = 19$ . Since genera of the basal lineages such as Palmeria and Hortonia also have  $x = 19$ , this is considered the base number of the family. Karyotype analyses showed that Hennecartia omphalandra, Kibaropsis caledonica, and Matthaea calophylla, for which we could analyze chromosome morphology, all had four chromosomes with a SC in the 38 somatic chromosomes. In addition, H. omphalandra had four long acrocentric chromosomes and four long submetacentric chromosomes; K. caledonica had four short acrocentric chromosomes; M. calophylla had four long submetacentric chromosomes and four short acrocentric chromosomes. In other words, the Monimiaceae often contain a set of four chromosomes with a SC in addition to sets of four (long or short) acrocentric and/or submetacentric chromosomes in  $2n = 38$  chromosomes (Tables 3–5). This suggests that  $2n = 38$  was derived by aneuploid reduction from  $2n = 40$ , a tetraploid of  $x = 10$ . Kibaropsis caledonica has the two longest acrocentric chromosomes, which may have resulted by chromosome fission, probably of two small chromosomes, and the subsequent fusion of chromosome fragments after  $2n = 40$  was established. It is also possible that reciprocal translocation, which occurred between two pairs of homologous chromosomes after  $2n = 38$  was established, may have resulted in such a long pair of chromosomes. Future studies should more critically analyze translocation in the chromosomes of K. caledonica.



The three genera (*Hennecartia*, *Kibaropsis*, and *Matthaea*) we examined had different karyotypes (or karyotype formulae). Their respective karyotype formula was  $2n = 38 = 26m + 4m^{SC} + 4sm + 4st$  in *Hennecartia*,  $2n = 38 = 26m + 2sm + 4sm^{SC} + 6st$  in *Kibaropsis*, and  $2n = 38 = 26m + 4sm + 4sm^{SC} + 4st$  in *Matthaea*. Like the four chromosomes with a SC, the four acrocentric and the four submetacentric chromosomes occupy different positions in all sequentially arranged chromosomes in different genera (Tables 3–5). These differences seem to have been generated by translocation between different chromosomes in individual genera.

#### The evolution of chromosomes in the Laurales

All chromosome data of the Laurales, including our original data, are summarized in Table 2. Except for several dubious counts, chromosome numbers are now available for 53 of 91–92 genera in the seven families. These data allowed us to determine base numbers for 39 genera without difficulty. The base numbers of individual genera are generally consistent within families:  $x = 11$  (Calycanthaceae);  $x = 12$  and  $15$  (Lauraceae);  $x = 19$  (Monimiaceae); and  $x = 22$  (Atherospermataceae and Siparunaceae). Exceptionally, the Hernandiaceae have diverse base numbers, i.e.,  $x = 15$  in *Gyrocarpus* and *Sparattanthelium* (Gyrocarpoideae),  $x = 18$  in *Illigera*, and  $x = 20$  in *Hernandia* (Hernandioideae). Noticeably, as will be discussed below, the groups with base numbers of  $x = 11$ ,  $22$ ,  $21$ , which indicate direct- or secondary direct-descendants from the ancestral stock of the Laurales, contain very few genera and species, whereas descendants via complex pathways to establish basic chromosome numbers, i.e.,  $12$ ,  $15$ ,  $19$ , are composed of very large numbers of genera and species.

Figure 19 shows a phylogenetic tree of the Laurales (using the Magnoliales as a sister group; Renner 1999; Qiu et al. 2005; Stevens 2005), on which chromosome data are mapped. The basic chromosome numbers of Magnoliales are

not yet clear. Particularly in the Myristicaceae, which are located in the most basal position within the order, diverse numbers such as  $x = 19, 21, 22, 25$ , and  $26$  are found (Oginuma and Tobe, unpublished data). While base numbers must be determined for the Myristicaceae and other Magnoliales in future studies, it is very likely that  $x = 11$  is an archaic base number in the Laurales and is still retained in the Calycanthaceae, which are sister to the remainder of the order. Polyploidization seems to have occurred from  $x = 11$  to  $x = 22$  in the common clade of the Atherospermataceae, Siparunaceae, and Gomortegaceae (although  $2n = 42$  in *Gomortega*). The  $2n = 42$  in *Gomortega nitida* may have resulted from an aneuploid decrease from  $2n = 44$ ; otherwise, this number must be reconfirmed, because 42 chromosomes represent a sufficiently large number to have been miscounted.

With regard to the chromosome evolution toward the common lineage of the Lauraceae, Hernandiaceae, and Monimiaceae, it is likely that an aneuploid decrease occurred from  $x = 11$  to  $x = 10$ . This suggestion is based on the fact that  $2n = 38$  in the Monimiaceae appears to have been derived by aneuploid reduction from  $2n = 40$ , a tetraploid of  $x = 10$ , as already stated. The  $x = 10$  occurs as a tetraploid (*Hernandia bivalis* and *H. ovigera*) in the Hernandioideae, although another genus, *Illigera* (*I. trifoliata*), has  $2n = 36$ . To understand the unusual number  $2n = 36$ , more species of *Illigera* must be investigated. However, the two other genera *Gyrocarpus* and *Sparattanthelium* (Gyrocarpoideae) are consistent in having  $x = 15$  ( $2n = 30$ ). They are probably of triploid origin from an ancestral lineage with  $x = 10$ .

The derivation of  $x = 12$  and  $15$  in the Lauraceae is most problematic. However, it seems likely that aneuploid increases occurred from  $x = 10$  to  $x = 12$  in an ancestral lineage of the family and further to  $x = 15$  probably independently in *Endiandra* and *Eusideroxylon* in the Cryptocaryeae because these two genera are not closely related (Chanderbali et al. 2001; Kimoto et al. 2006). With regard to their karyotypes, *Endiandra* has  $2n = 30 = 20m + 10sm$  (Oginuma et al. 1999), and

Eusideroxylon  $2n = 30 = 24m + 6sm$  (Heo and Oginuma 1994). In other genera of the Cryptocaryeae, Beilschmiedia, Cassytha, and Cryptocarya have  $x = 12$  (Table 2), but no records on the chromosomes of Aspidostemon, Caryodaphnopsis, Hypodaphnis, Potameia, and Potoxylon are currently available. Studies are therefore needed of more genera and species in the Cryptocaryeae to clarify chromosome evolution in the Lauraceae.

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## Legends of figures:

Figs. 1–6. Somatic chromosomes at metaphase in the Hernandiaceae. Figures 2, 4, and 6 are drawings of respective preceding photographs; 1, 2. Hernadia bivalis ( $2n = 40$ ); 3, 4. Hernadia ovigera ( $2n = 40$ ); 5, 6. Illigera trifoliata ( $2n = 36$ ). Arrows indicate chromosomes with a centromere at a subterminal position, arrowheads indicate chromosomes with a centromere at a submedian position, and white arrowheads indicate chromosomes with a secondary constriction. Scale bar = 2  $\mu\text{m}$ .

Figs. 7–12. Somatic chromosomes at metaphase in the Hernandiaceae, Lauraceae, and Monimiaceae. Figures 8, 10, and 12 are drawings of respective preceding photographs; 7, 8. Sparattanthelium amazonum ( $2n = 30$ ; Hernandiaceae); 9, 10. Cryptocarya laevigata ( $2n = 24$ ; Lauraceae); 11, 12. Hennecartia omphalandra ( $2n = 38$ ; Monimiaceae). Arrows indicate chromosomes with a centromere at a subterminal position, arrowheads indicate chromosomes with a centromere at a submedian position, white arrows indicate the longest chromosomes, and white arrowheads indicate chromosomes with a secondary constriction. Scale bar = 2  $\mu\text{m}$ .

Figs. 13–18. Somatic chromosomes at metaphase in the Monimiaceae. Figures 14, 16, and 18 are drawings of respective preceding photographs; 13, 14. Kibara macrophylla ( $2n = 114$ ); 15, 16. Kibaropsis caledonica ( $2n = 38$ ); 17, 18. Matthaea calophylla ( $2n = 38$ ). Arrows indicate chromosomes with a centromere at a subterminal position, arrowheads indicate chromosomes with a centromere at a submedian position, white arrows indicate the longest chromosomes, and white arrowheads indicate chromosomes with a secondary constriction. Scale

bar = 2  $\mu$ m.

Fig. 19. Evolution of chromosomes in the Laurales. The phylogenetic tree was drawn following Renner (1999), Stevens (2005), and Qiu et al. (2005). Triangles indicate that polyploidization occurred in that lineage. See text for further explanation.



Table 1. Taxa studied of Hernandiaceae, Lauraceae and Monimiaceae, and their collections and chromosome data

Taxon	Collection	Chromosome number and karyotype formula
<b>Hernandiaceae</b>		
<i>Hernandia bivalis</i> Benth.	Cultivated, Bogor Bot. Gard., Indonesia. <u>Tobe &amp; Oginuma 1304</u> (KYO).	$2n = 40 = 32m + 4sm + 4st$
<i>H. ovigera</i> L.	Cultivated, Bogor Bot. Gard., Indonesia. <u>Tobe &amp; Oginuma 1303</u> (KYO).	$2n = 40 = 32m + 4sm + 4st$
<i>Illigera trifoliata</i> Dunn	Thailand. Chiang Mai. <u>Maxwell 88-290</u> (Herbarium of Chiang Mai Univ.).	$2n = 36 = 28m + 2m^{sc} + 2sm + 4st$
<i>Sparattanthelium amazonum</i> Pilg.	Mexico. Selva Lacandona, Chajul. <u>Tobe &amp; Oginuma 346</u> (KYO).	$2n = 30 = 22m + 2sm + 6st$
<b>Lauraceae</b>		
<i>Cryptocarya laevigata</i> Blume	Cultivated, Kyoto University; grown from seeds obtained from North Coast Regional Botanic Garden, Australia. <u>Tobe 1335</u> (KYO).	$2n = 24 = 22m + 2sm$
<b>Monimiaceae</b>		
<i>Hennecartia omphalandra</i> Poisson	Paraguay. <u>Zardini &amp; Franco 54767</u> (MO).	$2n = 38 = 26m + 4m^{sc} + 4sm + 4st$
<i>Kibara macrophylla</i> (R. Cunn.) Benth.	Cultivated, Bogor Bot. Gard., Indonesia. <u>Tobe &amp; Oginuma 1334</u> (KYO).	$2n = 114$
<i>Kibaropsis caledonica</i> (Guillaumin) Jeremie	New Caledonia. Sarra-maea. <u>Kimoto s.n.</u> (KYO).	$2n = 38 = 26m + 4sm^{sc} + 2sm + 6st$
<i>Matthaea calophylla</i> Perkins	Cultivated, Bogor Bot. Gard., Indonesia. <u>Tobe &amp; Oginuma 1329</u> (KYO).	$2n = 38 = 26m + 4sm^{sc} + 4sm + 4st$

Table 2. Chromosome number and basic chromosome number of genera in Laurales

Family/genus <sup>1)</sup>	Total number of species	Number of examined species	Chromosome numbers <sup>2)</sup>		Base number
Atherospermataceae (6-7/16)					
<u>Atherosperma</u>	1	1	n = 21		x = ?
<u>Daphnandra</u>	6	1		2n = 44	x = 22
<u>Doryphora</u>	2	1	n = ca. 40	2n = ca. 82	x = ?
<u>Laurelia</u>	2	1		2n = 44	x = 22
Calycanthaceae (5/11)					
<u>Calycanthus</u>	2	2	n = 11	2n = 22, (24), 33	x = 11
<u>Chimonanthus</u>	6	3		2n = 22, 33	x = 11
<u>Idiospermum</u>	1	1		2n = 22	x = 11
<u>Sinocalycanthus</u>	1	1		2n = 22	x = 11
Gomortegaceae (1/1)					
<u>Gomortega</u>	1	1		2n = 42	x = 21
Hernandiaceae (5/57)					
<u>Gyrocarpus</u>	3	1	n = 15	2n = 30	x = 15
<u>Hernandia</u>	22	6	n = 20, 40	2n = 40, ca. 80	x = 20
<u>Illigera</u>	18	1		2n = 36	x = 18
<u>Sparattanthelium</u>	13	2		2n = 30, ca.48, ca.96	x = 15
Lauraceae (ca. 50/2500)					

<u>Actinodaphne</u>	100	3	$n = 12$	$2n = 24$	$x = 12$
<u>Aiouea</u>	21	1		$2n = 24$	$x = 12$
<u>Alseodaphne</u>	50	3	$n = 12$	$2n = 24$	$x = 12$
<u>Aniba</u>	41	1		$2n = 22, 24$	$x = ?$
<u>Apollonias</u>	1	1		$2n = 36, 48$	$x = 12$
<u>Beilschmiedia</u>	250	9	$n = 12, 24$	$2n = 24$	$x = 12$
<u>Cassytha</u>	20	2	$n = 12, 24$	$2n = 48$	$x = 12$
<u>Cinnamomum</u>	350	21	$n = (10), 12$	$2n = 24$	$x = 12$
<u>Cryptocarya</u>	200	2	$n = 12$	$2n = 24$	$x = 12$
<u>Endiandra</u>	100	1		$2n = 30$	$x = 15$
<u>Eusideroxylon</u>	1	1		$2n = 30$	$x = 15$
<u>Laurus</u>	1-2	2		$2n = 36, 42, 48, 54, 60, 66, 72$	$x = ?$
<u>Licaria</u>	40	1		$2n = 22, 24$	$x = ?$
<u>Lindera</u>	100	15	$n = 12$	$2n = 24$	$x = 12$
<u>Litsea</u>	400	24	$n = 12, 24$	$2n = 24$	$x = 12$
<u>Mezilaurus</u>	20	11		$2n = 22, 24$	$x = ?$
<u>Neolitsea</u>	100	3	$n = 24, 48$	$2n = 24, 48, 72$	$x = 12$
<u>Nothaphoebe</u>	40	1		$2n = 24$	$x = 12$
<u>Ocotea</u>	350	5	$n = 12$	$2n = 22, 24$	$x = ?$
<u>Persea</u>	200	18	$n = 12$	$2n = 24, 36, 48$	$x = 12$
<u>Phoebe</u>	100	8	$n = 12$	$2n = 24$	$x = 12$
<u>Sassafras</u>	3	2		$2n = 24, 48$	$x = 12$
<u>Umbellularia</u>	1	1		$2n = 24$	$x = 12$
<hr/>					
Monimiaceae (24/270)					
<u>Ehippiandra</u>	6	1		$2n = 42$	$x = ?$

<i>Hedycarya</i>	11	4	$n = 19$	$2n = 38, 114$	$x = 19$
<i>Hennecartia</i>	1	1		$2n = 38$ , ca. 96	$x = 19$
<i>Hortonia</i>	3	2		$2n = 38$	$x = 19$
<i>Kibara</i>	45	2		$2n = (44), 114$	$x = 19$
<i>Kibaropsis</i>	1	1	$n = 19$	$2n = 38$	$x = 19$
<i>Levieria</i>	7	1		$2n = 38$	$x = 19$
<i>Matthaea</i>	5	1		$2n = 38$	$x = 19$
<i>Mollinedia</i>	90	4		$2n = 36, 38, 180$	$x = ?$
<i>Monimia</i>	3	1	$n = \text{ca. } 44, \text{ ca. } 48$		$x = ?$
<i>Palmeria</i>	14	1		$2n = 38$	$x = 19$
<i>Peumus</i>	1	1		$2n = 78$	$x = ?$
<i>Tambourissa</i>	45	10	$n = 19$	$2n = 38$	$x = 19$
<i>Tetrasynandra</i>	3	1		$2n = \text{ca. } 86$	$x = ?$
<i>Wilkiea</i>	6	1		$2n = \text{ca. } 76$	$x = ?$
<i>Xymalos</i>	1	1		$2n = 38, 40-42$	$x = ?$

Siparunaceae (2/75)

<i>Siparuna</i>	74	3	$n = 22, 44$	$2n = 44, 88$	$x = 22$
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<sup>1)</sup> Familial circumscription and classification followed Renner (1999). The number of genera and species is presented in bracket.

<sup>2)</sup> Data from the book titled 'Chromosome numbers of flowering plants' (Fedorov 1974) and serial publication and book titled 'Index to plant chromosome numbers' (Ornduff 1967-1968; Moore 1973-1977; Goldblatt 1981-1988; Goldblatt and Johnson 1990-2003). Numbers that see rare are presented in bracket. Original or reconfirmed data published in this paper are indicated by bold.



Table 3. Measurements of 38 somatic chromosomes at metaphase in *Hennecartia omphalandra*. For explanation of individual values see materials and methods.

Chromosome	Length ( $\mu$ m)					Relative	Arm ratio	Shape
						length (%)		
1	0.4	+	1.4	=	1.8	4.2	3.5	st
2	0.4	+	1.4	=	1.8	4.2	3.5	st
3	0.4	+	1.3	=	1.7	3.9	3.3	st
4	0.4	+	1.3	=	1.7	3.9	3.3	st
5	0.5	+	1.1	=	1.6	3.7	2.2	sm
6	0.4	+	0.9	=	1.3	3.0	2.3	sm
7	0.5	+	0.9	=	1.4	3.2	1.8	sm
8	0.5	+	0.9	=	1.4	3.2	1.8	sm
9	0.6	+	0.2 + 0.6	=	1.4	3.2	1.3	m <sup>sc</sup>
10	0.6	+	0.2 + 0.6	=	1.4	3.2	1.3	m <sup>sc</sup>
11	0.6	+	0.2 + 0.6	=	1.4	3.2	1.3	m <sup>sc</sup>
12	0.6	+	0.2 + 0.6	=	1.4	3.2	1.3	m <sup>sc</sup>
13	0.6	+	0.8	=	1.4	3.2	1.3	m
14	0.6	+	0.8	=	1.4	3.2	1.3	m
15	0.5	+	0.7	=	1.2	2.7	1.4	m
16	0.5	+	0.7	=	1.2	2.7	1.4	m
17	0.5	+	0.6	=	1.1	2.5	1.2	m
18	0.5	+	0.6	=	1.1	2.5	1.2	m
19	0.5	+	0.6	=	1.1	2.5	1.2	m
20	0.5	+	0.6	=	1.1	2.5	1.2	m
21	0.4	+	0.6	=	1.0	2.4	1.5	m
22	0.4	+	0.6	=	1.0	2.4	1.5	m
23	0.5	+	0.5	=	1.0	2.4	1.0	m
24	0.5	+	0.5	=	1.0	2.4	1.0	m
25	0.4	+	0.5	=	0.9	2.0	1.3	m
26	0.4	+	0.5	=	0.9	2.0	1.3	m
27	0.4	+	0.5	=	0.9	2.0	1.3	m
28	0.4	+	0.5	=	0.9	2.0	1.3	m
29	0.4	+	0.5	=	0.9	2.0	1.3	m
30	0.4	+	0.5	=	0.9	2.0	1.3	m
31	0.4	+	0.5	=	0.9	2.0	1.3	m
32	0.4	+	0.5	=	0.9	2.0	1.3	m
33	0.4	+	0.4	=	0.8	1.9	1.0	m
34	0.4	+	0.4	=	0.8	1.9	1.0	m
35	0.4	+	0.4	=	0.8	1.9	1.0	m
36	0.4	+	0.4	=	0.8	1.9	1.0	m
37	0.3	+	0.3	=	0.6	1.4	1.0	m
38	0.3	+	0.3	=	0.6	1.4	1.0	m

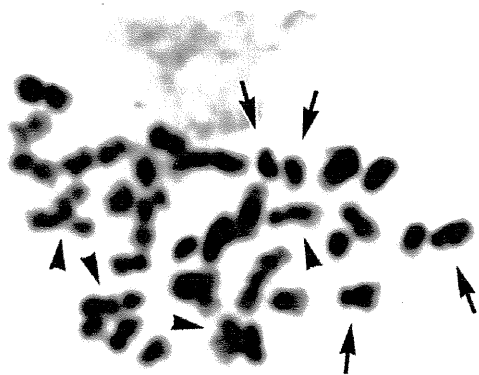
Table 4. Measurements of 38 somatic chromosomes at metaphase in *Kibaropsis caledonica*. For explanation of individual values see materials and methods.

Chromosome	Length ( $\mu\text{m}$ )					Relative	Arm ratio	Shape
						length (%)		
1	0.6	+	1.9	=	2.5	4.8	3.2	st
2	0.5	+	1.6	=	2.1	4.0	3.2	st
3	0.5	+	1.1	=	1.6	3.0	2.2	sm
4	0.5	+	1.1	=	1.6	3.0	2.2	sm
5	0.6	+	1.0	=	1.6	3.0	1.6	m
6	0.6	+	1.0	=	1.6	3.0	1.6	m
7	0.7	+	0.9	=	1.6	3.0	1.3	m
8	0.8	+	0.8	=	1.6	3.0	1.0	m
9	0.5	+	0.5 + 0.6	=	1.6	3.0	2.2	sm <sup>sc</sup>
10	0.5	+	0.5 + 0.6	=	1.6	3.0	2.2	sm <sup>sc</sup>
11	0.5	+	0.5 + 0.5	=	1.5	2.8	2.0	sm <sup>sc</sup>
12	0.5	+	0.5 + 0.5	=	1.5	2.8	2.0	sm <sup>sc</sup>
13	0.7	+	0.8	=	1.5	2.8	1.1	m
14	0.7	+	0.8	=	1.5	2.8	1.1	m
15	0.6	+	0.8	=	1.4	2.7	1.3	m
16	0.6	+	0.8	=	1.4	2.7	1.3	m
17	0.7	+	0.7	=	1.4	2.7	1.0	m
18	0.7	+	0.7	=	1.4	2.7	1.0	m
19	0.6	+	0.8	=	1.4	2.7	1.3	m
20	0.6	+	0.8	=	1.4	2.7	1.3	m
21	0.6	+	0.7	=	1.3	2.5	1.2	m
22	0.6	+	0.7	=	1.3	2.5	1.2	m
23	0.6	+	0.6	=	1.2	2.3	1.0	m
24	0.6	+	0.6	=	1.2	2.3	1.0	m
25	0.6	+	0.6	=	1.2	2.3	1.0	m
26	0.6	+	0.6	=	1.2	2.3	1.0	m
27	0.5	+	0.7	=	1.2	2.3	1.4	m
28	0.5	+	0.7	=	1.2	2.3	1.4	m
29	0.5	+	0.7	=	1.2	2.3	1.4	m
30	0.5	+	0.7	=	1.2	2.3	1.4	m
31	0.5	+	0.6	=	1.1	2.1	1.2	m
32	0.5	+	0.6	=	1.1	2.1	1.2	m
33	0.5	+	0.6	=	1.1	2.1	1.2	m
34	0.5	+	0.6	=	1.1	2.1	1.2	m
35	0.2	+	0.9	=	1.1	2.1	4.5	st
36	0.2	+	0.9	=	1.1	2.1	4.5	st
37	0.2	+	0.8	=	1.0	1.9	4.0	st
38	0.2	+	0.8	=	1.0	1.9	4.0	st

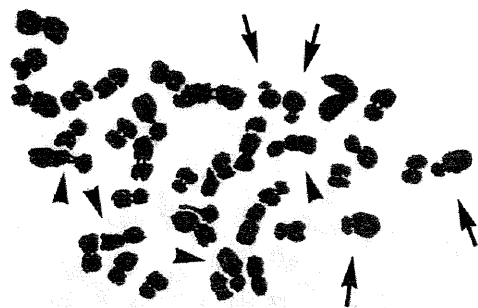
Table 5. Measurements of 38 somatic chromosomes at metaphase in *Matthaea calophylla*. For explanation of individual values see materials and methods.

Chromosome	Length ( $\mu\text{m}$ )				Relative length (%)	Arm ratio	Shape
1	0.9	+	0.7 + 0.9	= 2.5	4.6	1.8	sm <sup>sc</sup>
2	0.9	+	0.7 + 0.9	= 2.5	4.6	1.8	sm <sup>sc</sup>
3	0.7	+	0.6 + 0.9	= 2.2	4.1	2.1	sm <sup>sc</sup>
4	0.7	+	0.6 + 0.9	= 2.2	4.1	2.1	sm <sup>sc</sup>
5	0.8	+	1.4	= 2.2	4.1	1.8	sm
6	0.7	+	1.4	= 2.1	3.8	2.0	sm
7	0.7	+	1.3	= 2.0	3.7	1.9	sm
8	0.6	+	1.2	= 1.8	3.3	2.0	sm
9	0.9	+	0.9	= 1.8	3.3	1.0	m
10	0.8	+	0.9	= 1.7	3.1	1.1	m
11	0.7	+	0.9	= 1.6	3.0	1.3	m
12	0.7	+	0.9	= 1.6	3.0	1.3	m
13	0.7	+	0.9	= 1.6	3.0	1.3	m
14	0.8	+	0.8	= 1.6	3.0	1.0	m
15	0.7	+	0.8	= 1.5	2.7	1.1	m
16	0.7	+	0.8	= 1.5	2.7	1.1	m
17	0.6	+	0.8	= 1.4	2.6	1.3	m
18	0.6	+	0.8	= 1.4	2.6	1.3	m
19	0.6	+	0.8	= 1.4	2.6	1.3	m
20	0.7	+	0.7	= 1.4	2.6	1.0	m
21	0.5	+	0.8	= 1.3	2.4	1.6	m
22	0.5	+	0.8	= 1.3	2.4	1.6	m
23	0.6	+	0.7	= 1.3	2.4	1.2	m
24	0.6	+	0.7	= 1.3	2.4	1.2	m
25	0.6	+	0.6	= 1.2	2.2	1.0	m
26	0.6	+	0.6	= 1.2	2.2	1.0	m
27	0.5	+	0.6	= 1.1	2.0	1.2	m
28	0.5	+	0.6	= 1.1	2.0	1.2	m
29	0.5	+	0.6	= 1.1	2.0	1.2	m
30	0.5	+	0.6	= 1.1	2.0	1.2	m
31	0.5	+	0.5	= 1.0	1.8	1.0	m
32	0.5	+	0.5	= 1.0	1.8	1.0	m
33	0.4	+	0.5	= 0.9	1.6	1.3	m
34	0.4	+	0.4	= 0.8	1.5	1.0	m
35	0.1	+	0.6	= 0.7	1.3	6.0	st
36	0.1	+	0.6	= 0.7	1.3	6.0	st
37	0.1	+	0.5	= 0.6	1.1	5.0	st
38	0.1	+	0.5	= 0.6	1.1	5.0	st

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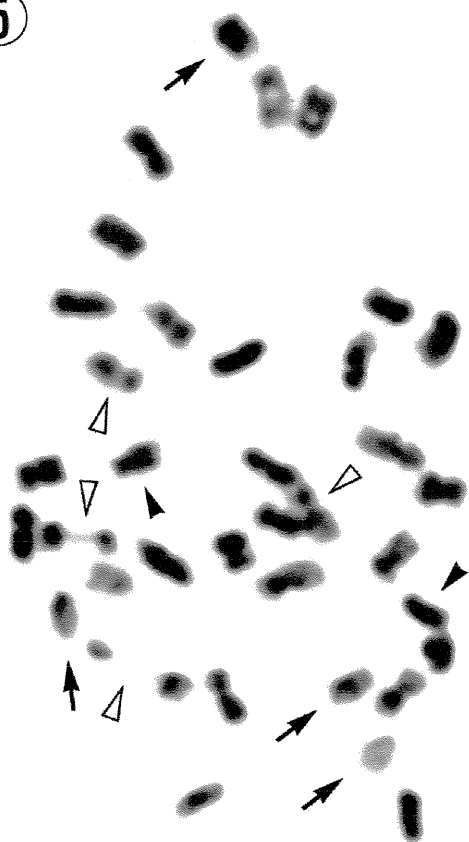
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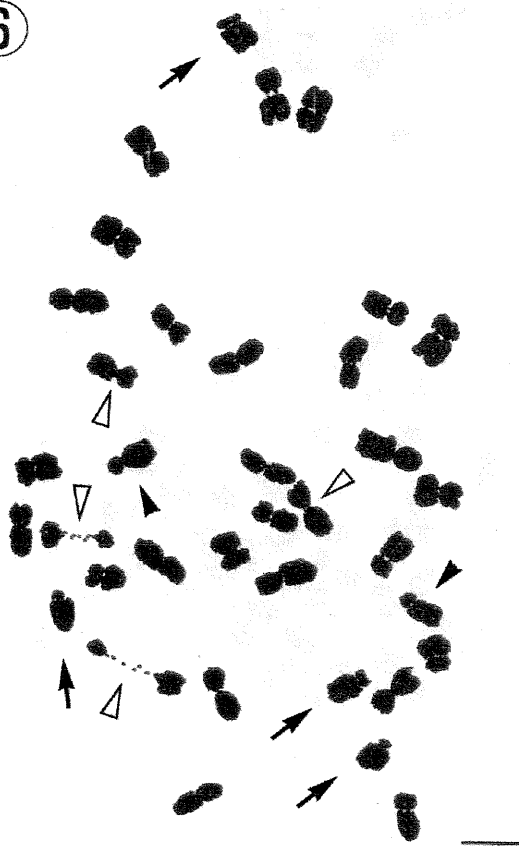
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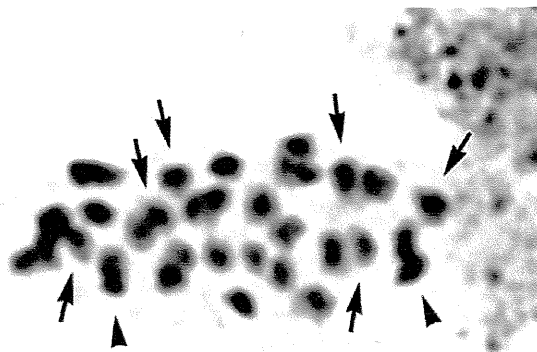


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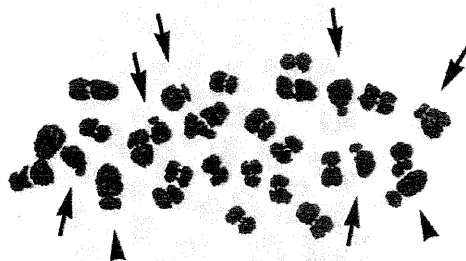




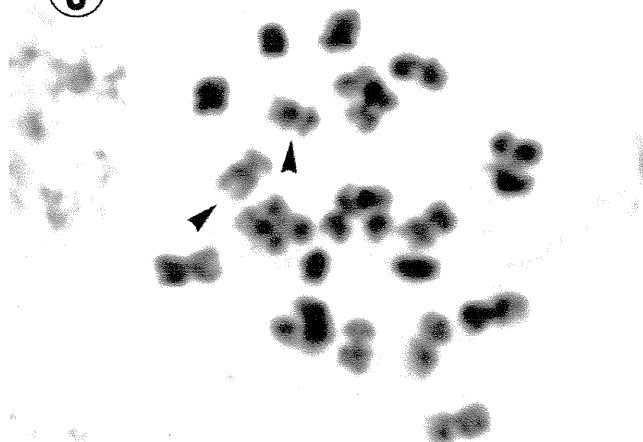
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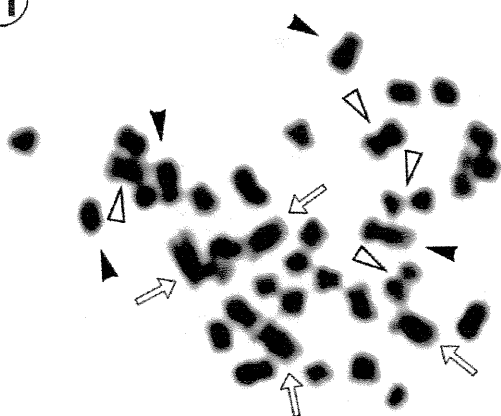
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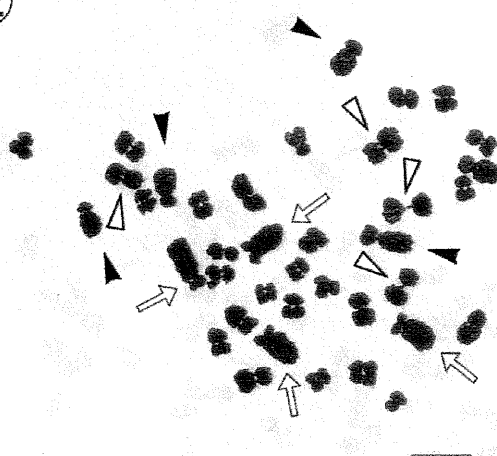
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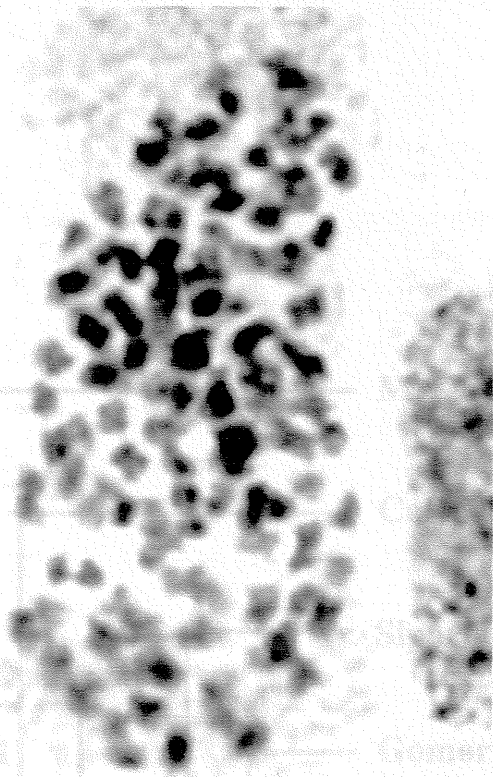
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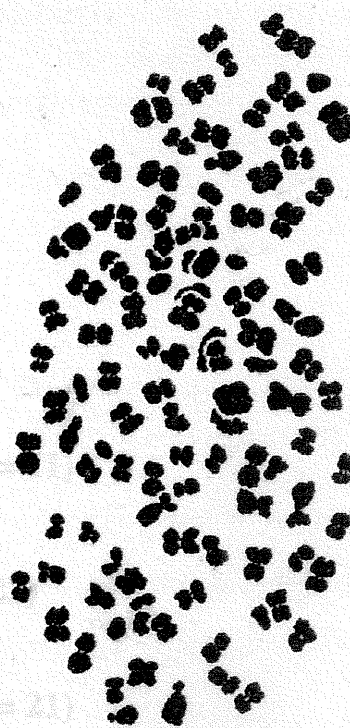
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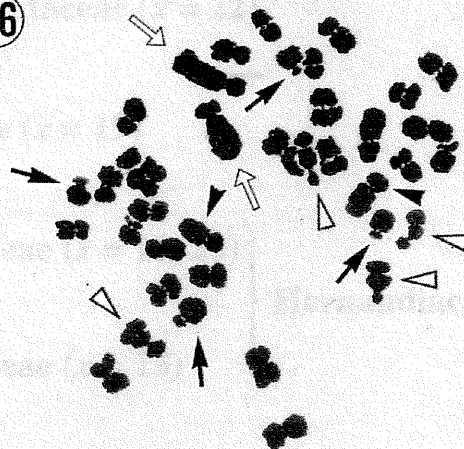
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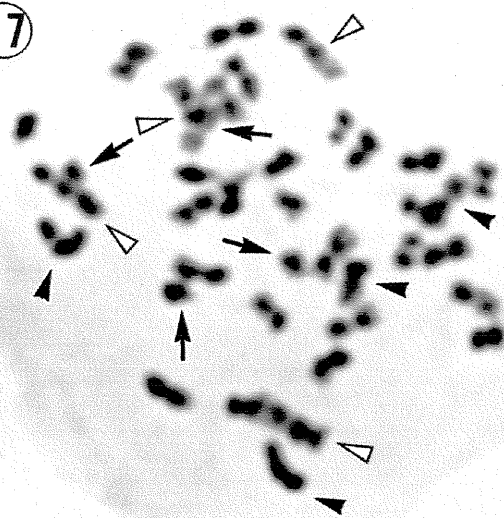
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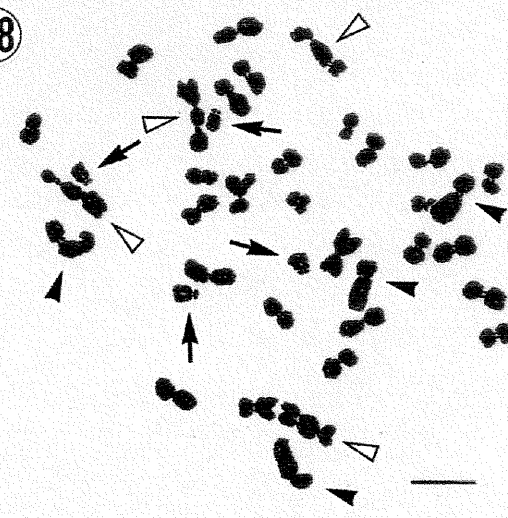
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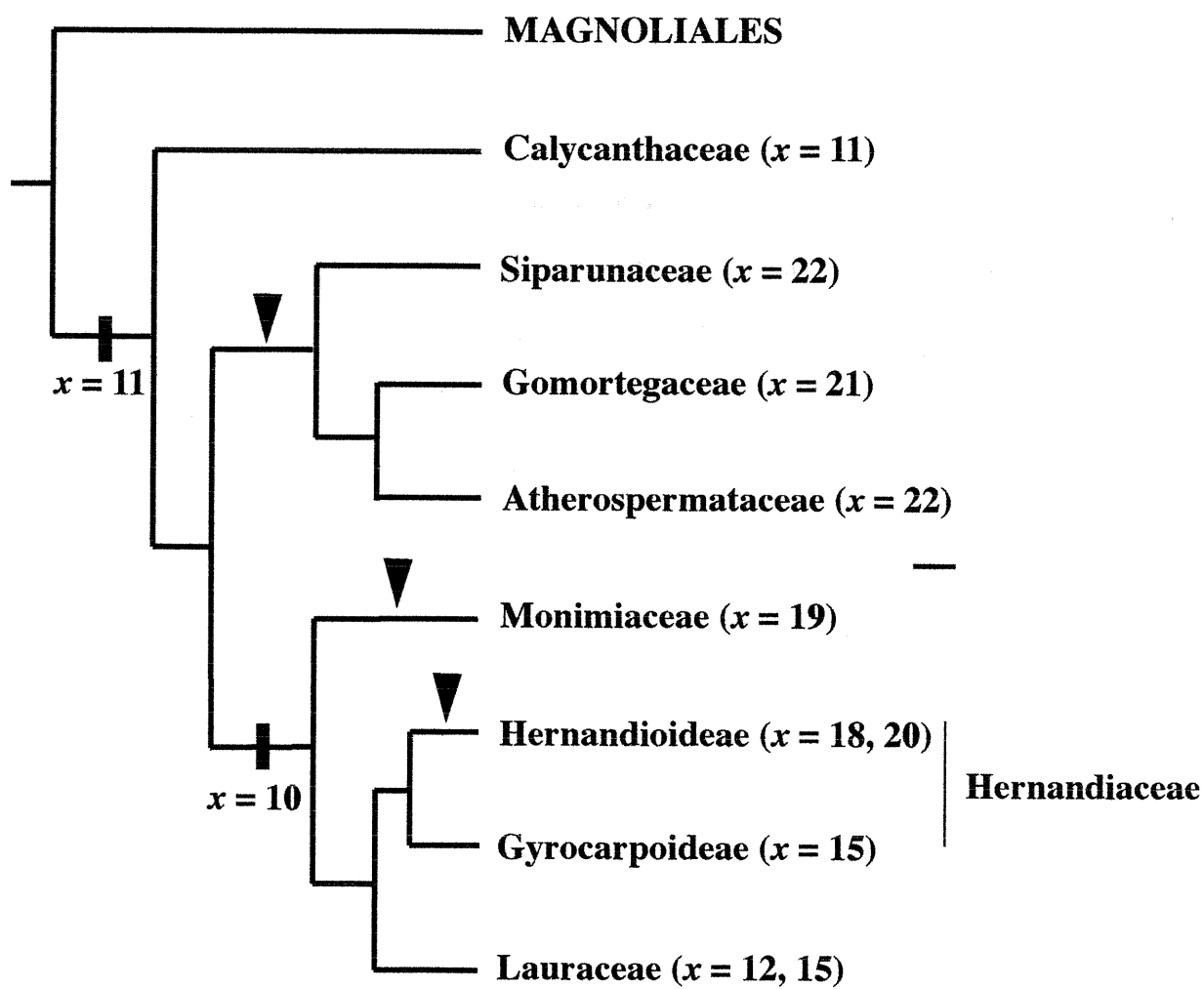


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**EMBRYOLOGY OF THE HORTONIOIDEAE AND MONIMIOIDEAE  
(MONIMIACEAE, LAURALES): CHARACTERISTICS OF LOWER  
MONIMIOIDS**

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Running title: Kimoto and Tobe - Embryology of the Monimiaceae



We investigated the embryology of the lower monimioids, i.e., the Monimioideae (Monimia, Palmeria, and Peumus) and Hortonioideae (Hortonia only), which are positioned basal within the Monimiaceae and are poorly described embryologically. Our results show that contrary to what has been reported in the literature, lower monimioids show very few variations in their embryological characters. Comparisons with the Mollinedioideae (a large derived subfamily in the Monimiaceae) and other families in the Laurales show that the lower monimioids are relatively consistent in sharing predominantly isobilateral tetrads of microspores, isobilateral tetrads of megaspores, a nonspecialized chalaza, and a mesotestal–endotestal seed coat (with tracheoidal cells of the meso- and endotesta). It is likely that while the shared successive cytokinesis in meiosis of microspore mother cells supports the Monimiaceae–Hernandiaceae–Lauraceae clade obtained by molecular evidence, no synapomorphies exist to support a sister-group relationship of the Monimiaceae with the Hernandiaceae or Lauraceae. Instead, the lack of hypostase, the lack of endosperm in mature seeds, and the amoeboid tapetum are likely synapomorphies of the Hernandiaceae and Lauraceae. For a critical comparison, however, studies of the Mollinedioideae and Hernandiaceae are needed, because embryological data for these families are insufficient.

**Keywords:** Embryology, Hernandiaceae, Lauraceae, Laurales, Monimiaceae

### Introduction

The Monimiaceae, comprising 25 genera and ca. 200 species distributed mainly in tropical regions of the Southern Hemisphere, are one of seven families of Laurales (Renner and Hausner 1997; Renner 1998, 1999). Until recently, the Monimiaceae were broadly circumscribed to include the Atherospermataceae and Siparunaceae (e.g., Money et al. 1950; Philipson 1993), which are now excluded as distinct families within the Laurales based on molecular evidence (Renner 1998, 1999). Molecular evidence has further shown that the Monimiaceae form a common clade with the Hernandiaceae and Lauraceae and are divided into three subfamilies, the Monimioideae (Monimia [3 spp.], Palmeria [15 spp.], and Peumus [1 sp.]), Hortonioideae (Hortonia [1–3 spp.]), and Mollinedioideae (Hedycarya and 20 other genera). Phylogenetically, the three genera of the Monimioideae diverged first, followed by Hortonia and the genera of the Mollinedioideae (Renner 1999, pers. comm.).

In a previous paper (Kimoto and Tobe 2001), we provided a review of earlier embryological studies of all seven families in the Laurales, noting that the Monimiaceae are poorly described embryologically. Although nine genera (Palmeria, Peumus, Hortonia, Austromatthaea, Hedycarya, Steganthera, Tambourissa, Tetrasynandra, and Wilkiea) have been relatively well studied embryologically (for review see Kimoto and Tobe 2001), none of them has ever been studied thoroughly. Eight other genera including Monimia have also been examined with respect to a few characters but still lack information on many other characters. Nevertheless, available embryological information has suggested that considerable variation is likely to exist in the Monimiaceae (Kimoto and Tobe 2001). For example, the number of microsporangia is four in most genera but two in Monimia; the mode of embryo sac formation conforms to the Polygonum type in Hedycarya aroborea but to the Allium type in Peumus boldus; antipodal cells are

ephemeral in most genera but are persistent and multiplied into five, eight, or more cells in Peumus; the micropyle is formed by the inner integument alone in Peumus, Hedycarya, Kibara, Wilkiea, and Tambourissa but by both integuments in Monimia, Kibaropsis, and Palmeria. More genera and species, particularly of the Monimioideae (because Peumus often shows features distinct from those of the other genera), must be studied to confirm the presence or absence of such embryological variations in the family, how individual genera or groups of genera are characterized embryologically, and how embryological characters have evolved within the family.

In this paper, we present an embryological study of all four genera of the lower or basal monimioids, i.e., the Monimioideae and Hortonioideae. Because of their basal positions in the phylogenetic tree of the Monimiaceae, they should provide a basis for comparison with higher monimioids (Mollinedioideae) and also with related families (Lauraceae and Hernandiaceae). This is our fourth paper on the embryology of Laurales subsequent to those of the Siparunaceae (Kimoto and Tobe 2003), Gomortegaceae (Heo et al. 2004), and Eusideroxylon (Lauraceae; Kimoto et al. 2006). Since additional embryological data are accumulating from other Laurales, we are now in a good position to discuss characteristics of the basal monimioids.

### Materials and Methods

In total, we investigated six species of Monimioideae and Hortonioideae: Monimia amplexicaulis Lorence, M. ovalifolia Thours, M. rotundifolia Thours, Palmeria scandens F. Muell., Peumus boldus Molina, and Hortonia floribunda Wight. ex Arn. Collection data and developmental stages for each species are presented in Table 1. Flower buds, flowers, and seeds in various stages of development were fixed with FAA

(five parts formalin, five parts acetic acid, 90 parts 50% ethanol). Some of the materials were dehydrated through a *t*-butyl alcohol series and embedded in Paraplast (57–58°C mp). Serial sections were cut to a 4–8 µm thickness with a rotary microtome, stained with Heidenhain's hematoxylin, safranin, and fastgreen FCF, and mounted with Entellan. To examine seed coat structure, which is enclosed by a hard endocarp, several young and mature fruits were embedded in Technovit 7100 (Kulzer, Germany) after dehydration through an ethanol series. Technovit resin was polymerized at 2 to 4°C for 12 to 48 h and kept at 60°C for 2 days. Serial sections cut to a thickness of 4 to 6 µm were stained with Heidenhain's hematoxylin and/or safranin (Kimoto and Tobe 2003).

To count the number of generative cells in a pollen grain at the time of shedding, mature pollen grains were stained with 1% acetocarmine (Tobe and Raven 1984). To observe vasculature in mature ovules and young seeds, they were bleached overnight at room temperature with 0.01% sodium hypochlorite (NaClO). After being washed with distilled water several times, they were stained with 1% safranin O for 2 days (Fukuhara 1992; Kimoto and Tobe 2003). Terminologies of seed coat structure followed Comer (1976) and Schmid (1986).

## Results

The following descriptions of embryological characters are common to all four genera investigated unless otherwise stated. Embryological features of each genus are summarized in Table 2.

### Anthers and Microspores

Flowers are unisexual except in Hortonia. A cup-shaped (male) flower bears three



or more stamens (and several staminodia in Hortonia) (Fig. 1A). Anthers are bisporangiate in Monimia (Fig. 1B,C) or tetrasporangiate in Hortonia, Palmeria, and Peumus as reported previously (Kimoto and Tobe 2001). Although it is uncertain how it is formed, the anther wall prior to maturation comprises five to eight cell layers: an epidermis, an endothecium, two to five middle layers, and a tapetum (Fig. 1D). The tapetum is glandular, and its cells become two-nucleate (Fig. 1E,F). In M. ovalifolia and M. rotundifolia, two tapetal nuclei are always fused into one nucleus. During maturation, epidermal cells become flattened (or collapsed in Monimia) and endothelial cells develop fibrous thickenings, while the middle layers degenerate. Thus the mature anther wall consists of the flattened (or collapsed) epidermis and the fibrous endothecium (Fig. 1G).

Meiosis in a microspore mother cell is accompanied by successive cytokinesis (Fig. 1H-J). After the first division, cytoplasm is divided into two sister cells (Fig. 1H), in each of which the second division occurs (Fig. 1I). The shape of a tetrad of microspores is predominantly isobilateral (Fig. 1J). Each anther is dehiscent by two longitudinal slits (Fig. 1C) as reported previously (see Kimoto and Tobe 2001). Pollen grains are two-celled at the time of shedding, although only the nucleus of the generative cell is stained by acetocarmine (Fig. 1K).

#### Ovule, Nucellus, and Megagametophyte

A cup-shaped female (or bisexual) flower usually has two to five (or more) carpels (Fig. 2A) as described previously (Endress 1980; Philipson 1993). Each carpel has a single ovule, which arises from an apical placenta in a locule and becomes anatropous with the micropyle upward (Fig. 3F) as already reported (Endress 1980). The ovule is crassinucellate. The archesporium is multicelled, comprising two or three cells (Fig. 2B) (data not available for Palmeria and Hortonia), of which usually one, and rarely two or

three, divides into a primary parietal cell and a primary sporogenous cell. The primary parietal cell further divides to form parietal tissue of three to eight cell layers (Fig. 2C-I). The primary sporogenous cell differentiates into a megaspore mother cell, which undergoes meiosis (Fig. 2C), successively resulting in a dyad of megaspores and an isobilateral tetrad of megaspores (Fig. 2D-G). In each tetrad, two megaspores derived from the upper cell of a dyad and two megaspores derived from the lower cell of the dyad are arranged horizontally, although they may be sometimes arranged somewhat obliquely (Fig. 2D-G). The two micropylar megaspores are generally smaller than the two chalazal megaspores. We observed such isobilateral tetrads in all nine examined ovules at the megaspore tetrad stage: in two ovules of Hortonia, three ovules of Peumus, three ovules of Monimia amplexicaulis, and one ovule of M. ovalifolia. Usually one tetrad is formed, but rarely twin tetrads are formed (Fig. 2D,G). While one of the two chalazal megaspores is functional, the other three are degenerate (Fig. 2H,I).

By undergoing nuclear divisions, the functional megaspore develops successively into a two- (Fig. 3A,B), four- (Fig. 3C), and eight-nucleate embryo sac. A mature embryo sac has an egg cell, two synergids, two polar nuclei, and three antipodal cells (Fig. 3D). Thus, the mode of embryo sac formation is of the Polygonum type not only in Hortonia, Monimia, and Palmeria, but also in Peumus (P. boldus), although Mauritzon (1935) reported the Allium type in P. boldus. Only one mature embryo sac is formed (Fig. 3D-F), which is ellipsoid or oblong-ellipsoid in shape. The antipodal cells degenerate soon after fertilization (Fig. 3E). In P. boldus, we could not observe the secondary cell division of antipodal cells, contrary to the description by Mauritzon (1935). No conspicuous starchy grains were observed in the embryo sac.

During megagametogenesis, a two-cell-layered nucellar cap is formed by periclinal divisions of epidermal cells of the nucellus (Fig. 2D,F). No nucellar beak was formed.

Nucellar tissue remains around the mature embryo sac (Fig. 3E) and even in young seeds.

No conspicuous obturator is formed.

### Integuments

The ovule is bitegmic (Figs. 3E, 4A-D), as already reported (Mauritzon 1935; Corner 1976; Endress 1980; Endress and Igersheim 1997). Neither the inner nor the outer integument is multiplicative. The inner integument is initiated by periclinal divisions of epidermal cells of the ovule primordium at the archesporial cell stage (and is thus of dermal origin), and is three to four cells thick throughout development (Fig. 4A). Soon after the initiation of the inner integument, the outer integument is initiated by cell divisions of both dermal and subdermal cells of the ovule primordium (and is thus of subdermal origin; Fig. 4A). The outer integument is three to five cells thick throughout development. Neither the inner nor the outer integument has vascular bundles (Fig. 4B). The vascular bundle running through the raphe ends in the chalaza (Fig. 4D), where it is ramified into three or more branches (Fig. 3E).

The micropyle is formed by the inner integument alone in Hortonia and Monimia. Although Endress and Igersheim (1997) described that the micropyle is formed by both the inner and outer integuments in Monimia and Palmeria, the tip of the outer integument does not grow beyond the tip of the inner integument (Fig. 4C: M. rotundifolia). Corner (1976, p. 333, Fig. 394) also provided a drawing illustrating that the micropyle is formed only by the inner integument in H. floribunda. In P. boldus, the micropyle is not formed because the nucellar apex is exposed (Fig. 3E), as reported by Endress and Igersheim (1997). No endothelium is formed.

### Endosperm and Embryo

Fertilization is porogamous except in Peumus (which does not form the micropyle). In Peumus as well, however, fertilization is undertaken by the pollen tube passing through the apex of the nucellus. The mode of endosperm formation is of ab initio cellular type (Fig. 4E), as reported previously for Peumus (Mauritzon 1935). Endosperm is abundant in mature seeds (Fig. 5B). Cells of the endosperm contain oil droplets (Fig. 4F). No rumination is observed. Embryogenesis was not investigated in detail, but a drawing of Peumus suggests the Onagrad type (Mauritzon 1935, p. 321, Fig. 2). Several microtome sections showed that embryogenesis proceeds normally (Fig. 5A). Embryos in mature seeds are straight and symmetrical with two thin cotyledons (Fig. 5B). A suspensor is not conspicuous throughout development.

### Seed and Seed Coat

Fruits are one-seeded drupes with a thick and hard endocarp. In young seeds, the hypostase is differentiated (Fig. 4E) as described by Corner (1976, p. 195) for Hortonia. The chalaza is small and does not exhibit any specialization (Figs. 3E, 4D) such as pachychalazy or perichalazy. Neither the size nor the shape of the chalaza changes throughout the development of the seed.

Mature seeds are exarillate and ovoid to widely ovoid in shape (Fig. 5B), and their sizes vary among genera. They are ca. 10 mm long and ca. 6 mm wide in Hortonia (Corner 1976), ca. 5 mm long and ca. 4 mm wide in Palmeria (Corner 1976), ca. 5–6 mm long and ca. 3–4 mm wide in Peumus, and ca. 3 mm long and ca. 2 mm wide in Monimia.

The seed coat is thin. Cells of the tegmen (i.e., developed inner integument) are enlarged early in development but completely crushed in later stages (Fig. 5C,D; also, compare Fig. 5E to Fig. 5F and Fig. 5G to Fig. 5H and subsequently to Fig. 5I). In



contrast, cells of the testa (i.e., developed outer integument) remain in mature seeds, forming a persistent structure three to five cell layers thick (Fig. 5D,E,I). At maturity, cells of the exotesta are thin-walled, whereas those of the mesotesta and endotesta develop annular thickenings on their radial walls and become tracheoidal (Fig. 5C,D,F,I). The seed coat is thus "mesotestal-endotestal."

## Discussion

### Summary of Embryological Features of Lower Monimioids

Embryological features of Monimia, Palmeria, and Peumus of the Monimioideae and Hortonia of the Hortonioideae are summarized in Table 2 along with those of the Mollinedioideae. As reported in our previous paper (Kimoto and Tobe 2001, pp. 251–252, Table 3), many of the embryological data in the Monimiaceae were missing. The present study has filled most of these gaps for the lower monimioids. Embryological features of the lower monimioids are summarized as follows.

Anther bi- (Monimia) or tetrasporangiate (Palmeria, Peumus, and Hortonia); mode of anther wall formation dicotyledonous; anther wall prior to maturation five to seven cell layered; anther epidermis flattened; endothecium fibrous; middle layers crushed; tapetum glandular and its cells essentially two-nucleate; cytokinesis in the microspore mother cell successive; microspore tetrads predominantly isobilateral in shape; mature pollen grains two-celled; anther dehiscing by longitudinal slits.

Ovule anatropous; ovule archesporium two- to three-celled; ovules crassinucellate; parietal tissue two to four cells thick; megaspore tetrad(s) isobilateral; embryo sac formation conforming to the Polygonum type; mature embryo sac single, and ellipsoid (or rarely oblong-ellipsoid) in shape; antipodal cells ephemeral; starchy grains not

conspicuous in mature embryo sacs; two-cell-layered nucellar cap formed; no nucellar beak formed; nucellar tissue remaining in mature ovules; obturator not formed.

Ovules bitegmic and not multiplicative; both integuments two to three (to five) cells thick throughout development. Histogenetically, inner integument of dermal origin and outer integument of subdermal origin; no vascular bundle(s) present in the integuments (or seed coats); micropyle formed by the inner integument alone (Monimia, Hortonia) or by the inner and outer integuments (Palmeria, although reconfirmation needed), but not formed in Peumus; no endothelium formed.

Fertilization porogamous when the micropyle is formed; endosperm formation of ab initio cellular type; embryogenesis conforming to the Onagrad type; proembryo with a short suspensor.

In seeds, hypostase formed; raphal vascular bundle ramifying at the chalaza; mature seed exarillate and albuminous; perisperm absent. Embryo straight with two cotyledons. Seeds not ruminated.

Seed coat not multiplicative; type of seed coat "mesotestal-endotestal," and tegmen crushed; cells of the mesotesta and endotesta tracheoidal.

#### Comparisons within the Monimiaceae

As summarized above, contrary to what was suggested in the Introduction on the basis of a literature survey, the lower monimioids show very few variations in their embryological characters. We confirmed that while the anther is tetrasporangiate in Palmeria, Peumus, and Hortonia, it is bisporangiate in Monimia as reported by Lorence (1985). In addition, while the micropyle is formed by the inner integument alone in Monimia and Hortonia, it is formed by the inner and outer integuments in Palmeria gracilis (Endress and Igersheim 1997) or not formed in Peumus. Except for these

differences, we did not observe variations in other characters such as the mode of embryo sac formation and the presence or absence of persistent antipodal cells (see Kimoto and Tobe 2001). Mauritzon (1935) reported the Allium type embryo sac in Peumus boldus, but we found that P. boldus has the Polygonum type embryo sac as in Monimia, Palmeria, and Hortonia. The Allium type is a term given to the mode forming a bisporic eight-nucleate embryo sac (Maheshwari 1950). We found that megasporogenesis nearly always resulted in an isobilateral tetrad of megaspores, not only in Monimia and Hortonia but also in Peumus (Fig. 2D–H). Usually the two cells derived from the upper cell at the preceding dyad stage were much smaller than the two cells derived from the lower cell. The former two cells may have escaped the observation of Mauritzon (1935), so that the latter two cells may have resembled cells of a dyad, with one of them developing into an embryo sac, as is observed when the Allium type embryo sac is formed. Mauritzon (1935, p. 322) further reported that "die Anzahl der Antipoden ist oft 5–8, aber nicht selten erreicht sie etwa 20" ("the number of antipodal cells is often 5–8, but can reach approximately 20"). However, neither persistent nor amplified antipodal cells were detected in our materials.

When we compared the embryological features of the lower monimioids to those of the Mollinedioideae, the following four characters stood out: predominant shape of the microspore tetrads, shape of the megaspore tetrad, specialization of the chalaza, and seed coat structure. While isobilateral tetrads of microspores are observed as predominant shapes in the lower monimioids, both isobilateral and decussate tetrads are common in the Mollinedioideae (see footnote 3 in Table 2). Likewise, while isobilateral tetrads of megaspores are prevalent in the lower monimioids, both linear and T-shaped, rather than isobilateral, tetrads have been reported in the Mollinedioideae (see footnote 5 in Table 2). Maheshwari (1937) summarized records of the isobilateral tetrads of megaspores, stating

that they occur only as abnormalities, as in Myrtus communis and Urginea indica. However, we found that the isobilateral tetrads of megaspores occur consistently and are characteristic features at least in the lower monimioids. The ovule had no specialized chalaza in the lower monimioids examined in our study, but it is likely perichalazal in the Mollinedioideae (see footnote 6 in Table 2). The seed coat is mesotestal–endotestal in the lower monimioids but endotestal in the Mollinedioideae (see footnote 7 in Table 2).

The Mollinedioideae remain very poorly described embryologically. The embryological features presented in Table 2 were derived from a very limited number of taxa (for a description of the poor state of knowledge of the Mollinedioideae, see Table 4 in Kimoto and Tobe 2001). In other words, it is not yet clear whether and how the largest and derived subfamily Mollinedioideae is diversified in embryological characters. We must investigate the Mollinedioideae to clarify how this subfamily differs from the lower monimioids in its embryological characters.

#### Comparisons to Other Lauralean Families

In this study, we have provided an overall picture of the embryological features of lower monimioids. Irrespective of embryological features of the higher monimioids, i.e., the Mollinedioideae, the results of this study provide a basis for comparison with other families of Laurales.

While filling in the missing data or revising the data in Table 3 of Kimoto and Tobe (2001), we have confirmed that the lower monimioids have many embryological characteristics in common with other lauralean families. They share the multicelled ovule archesporium (as a synapomorphy of the Laurales) with all other lauralean families except the Gomortegaceae (Heo et al. 2004). Molecular analyses have shown that the Monimiaceae, Hernandiaceae, and Lauraceae form a common clade within the Laurales



(Renner and Chanderbali 2000). We have pointed out that successive cytokinesis in meiosis of a pollen mother cell may be a synapomorphy of the three families (Kimoto and Tobe 2001). We have confirmed that all four genera of the lower monimioids show successive cytokinesis. However, since successive cytokinesis is also seen in the Siparunaceae (Kimoto and Tobe 2003), it is likely a homoplasy supporting the Monimiaceae–Hernandiaceae–Lauraceae clade. The exact position of character-state evolution on the phylogenetic tree will be discussed again when we have completed our studies of the Atherospermataceae and Hernandiaceae, which are yet poorly described embryologically.

Molecular analyses have not yet resolved the relationships within the Monimiaceae–Hernandiaceae–Lauraceae clade because of the lack of phylogenetically informative substitutions (Renner and Chanderbali 2000). The three families are different in basic chromosome number, i.e.,  $\underline{x} = 19$  in the Monimiaceae,  $\underline{x} = 10, 15$ , and 18 in the Hernandiaceae, and  $\underline{x} = 12$  and 15 in the Lauraceae (Oginuma and Tobe 2006). Karyotype analyses suggest that aneuploid reduction from  $\underline{x} = 11$  to  $\underline{x} = 10$  is likely to have occurred in the common clade of the Monimiaceae, Hernandiaceae, and Lauraceae (Oginuma and Tobe 2006); however, no synapomorphies were found suggesting relationships among the three families. However, Doyle and Endress (2000, pp. 133–134) enumerated morphological synapomorphies of the Hernandiaceae and Lauraceae, such as solitary vessels, simple perforations, well developed paratracheal parenchyma, mucilage cells, richly branched inflorescences, two perianth whorls, granular infratectal structure, reduced tectum, one carpel, multilayered pollen tube transmitting tissue, outer integument with four to many cell layers, multiplicative testa, large embryo, no endosperm in the mature seed, and hypogeal germination. Previous studies have also noted that closer relationships are supported by embryological evidence

between the Hernandiaceae and Lauraceae than between either one of them and the Monimiaceae (Heo and Tobe 1995; Heo et al. 1998; Kimoto and Tobe 2001). In addition to the exalbuminous seeds, we suggested that the lack of hypostase and the anther dehiscence mode by valves were synapomorphies of the Hernandiaceae and Lauraceae. However, since the dehiscence mode by valves is known in the Atherospermataceae, Gomortegaceae, and Siparunaceae (Kimoto and Tobe 2001, 2003; Heo et al. 2004), it may be a synapomorphy of a larger or basal clade. We instead suggest the amoeboid tapetum as another synapomorphy of the Hernandiaceae and Lauraceae.

The Monimiaceae, at least the lower monimioids, have several autapomorphies. As we have already pointed out (Kimoto and Tobe 2001), the non-multiplicative testa (a homoplasy), the mode of anther dehiscence by longitudinal slits (a reversal), and the non-crushed mesotesta (a reversal) are autapomorphies, making them different from both the Hernandiaceae and Lauraceae. In addition, the predominant occurrence of isobilateral microspore tetrads and isobilateral megaspore tetrads, as well as the nonspecialized chalaza and the mesotestal-endotestal seed coat, are likely autapomorphies of the Monimiaceae. Before concluding what autapomorphies exist in the Monimiaceae, however, we must await results of investigations on the Hernandiaceae, which are not well described, although the family appears to be diversified in its embryological characters.

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## Legends for figures

**Fig. 1** Development of anthers and microspores in the Monimioideae. A, H, Monimia ovalifolia. B, D, Monimia amplexicaulis. C, E, F, I, J, Monimia rotundifolia. G, Peumus boldus. A, Longitudinal section (LS) of a male flower bud. B, Transverse section (TS) of a young anther. C, LS of an old stamen. An arrow points out the area where a longitudinal slit is formed. D, TS of a young anther showing anther wall structure. E, Two-nucleate tapetum. F, TS of an old anther, showing glandular tapetum. G, TS of a mature anther. H, Meiosis I in a microspore mother cell. Arrow indicates the cell wall formed by the first meiotic division. I, Meiosis II in a microspore mother cell. Arrow indicates the cell wall as in H. J, Microspore tetrad. K, Mature pollen stained with acetocarmine. ep = epidermis; et = endothecium; g = generative cell; ml = middle layer; p = pollen; pmc = microspore mother cell; st = stamen; t = tapetum. A, bar = 1 mm; B, bar = 100  $\mu$ m; C, bar = 200  $\mu$ m; D, E, G-K, bar = 20  $\mu$ m; F, bar = 50  $\mu$ m.

**Fig. 2** Development of megagametophytes in the Hortonioideae and Monimioideae. A, F, Monimia ovalifolia. B, G, Monimia amplexicaulis. C, D, H, Peumus boldus. E, I, Hortonia floribunda. All sections are longitudinal sections of a flower or ovules. A, Open female flower. B, Young ovule with archesporial cells. C, Ovule with a megaspore mother cell in meiosis. D-G, Ovules with a tetrad(s) of megaspores. Arrows (and arrowheads) indicate four megaspores in a tetrad. Note that twin tetrads of megaspores are formed in D and G. H, I, Ovules in which one megaspore is functional with three degenerating megaspores (arrows). arc = archesporial cell; fc = functional megaspore; mmc = megaspore

mother cell; nc = nucellar cap; ov = ovule; pl = carpel; pt = parietal tissue. A,

bar = 1 mm; B-I, bar = 20  $\mu$ m.

**Fig. 3** Development of embryo sacs in the Monimioideae. A, C-F, Peumus boldus. B, Monimia rotundifolia. All sections are longitudinal sections of ovules. A, Two-nucleate embryo sac with degenerating megaspores (arrows). B, Two-nucleate embryo sac. C, Four-nucleate embryo sac. D, Mature embryo sac. E, Embryo sac just after fertilization. F, Mature ovule showing the whole structure. ant = antipodal cell; ch = chalaza; e = egg cell; es = embryo sac; ii = inner integument; n = nucleus of an embryo sac; nu = nucellus; pen = primary endosperm nucleus; po = polar nucleus; syn = synergid; vs = vascular bundle; z = zygote. A, B, bar = 20  $\mu$ m; C-E, bar = 50  $\mu$ m; F, bar = 100  $\mu$ m.

**Fig. 4** Development of integuments and the endosperm in Monimioideae. A, B, F, Peumus boldus. C, D, Monimia rotundifolia. E, Monimia ovalifolia. A, Longitudinal section (LS) of a young ovule showing developing integuments. Asterisks (\*) indicate cells derived from dermal cells in an ovule primordium. B, Transverse section of a mature ovule. C, LS of a mature ovule showing the micropyle. D, Cleared mature ovule stained with safranin. Arrows indicate junctions between the nucellus and the inner integument and between the inner integument and the outer integument. E, LS of a young seed with the cellular endosperm. F, Endosperm in mature seeds. ch = chalaza; en = endosperm; es = embryo sac; hyp = hypostase; ii = inner integument; mic = micropyle; nu = nucellus; oi = outer integument; r = a vascular bundle in the raphe. A, C, bar = 50  $\mu$ m; B, D, E, bar = 100  $\mu$ m; F, bar = 20  $\mu$ m.

**Fig. 5** Development of embryos and seed coats in the Monimioideae and Hortonioideae.

A, E, F, Monimia ovalifolia. B, Palmeria scandens. C, D, Hortonia floribunda. G-I, Peumus boldus. A, Longitudinal section (LS) of a proembryo. B, LS of a mature seed. C, E, G, LS of a young fruit at the antiraphal side. D, F, H, LS of a nearly mature seed showing the structure of the antiraphal side. I, LS of a mature seed showing the structure of the raphal side. emb = embryo; en = endosperm; encl = endocarp; ents = endotesta; exts = exotesta; msts = mesotesta; nu = nucellus; pem = proembryo; s = suspensor; tg = tegmen; ts = testa. A, bar = 20  $\mu$ m; B, bar = 1 mm; C, D, bar = 50  $\mu$ m; E, G, H, bar = 100  $\mu$ m; F, I, bar = 20  $\mu$ m.

**Footnotes of Table 2**

Abbreviations: ii, inner integument; oi, outer integument.

- 1) In the two Monimia species (M. ovalifolia and M. rotundifolia) that were observed for this character, the tapetum was two-nucleate in the beginning, although the two tapetal nuclei were always fused into one nucleus.
- 2) According to Foreman (1984, pp. 129–130), microspore tetrads are “mostly isobilateral, decussate or occasionally intermediate between these two forms” in Palmeria coriacea, and “usually isobilateral or decussate with a few intermediate forms also being seen” in P. scandens.
- 3) Foreman (1984) reported that both isobilateral and decussate tetrads of microspores are found in Austromatthaea elegans, Hedycarya angustifolia, H. loxocarya, Steganthera



macrooraia, Tetrasyandra laxiflora, Wilkiea austroqueenslandica, and W. hugeliana.

Regarding W. austroqueenslandica, Foreman (1984, p. 133) stated that the "tetrads are mainly isobilateral, decussate or intermediate between these two forms, but linear and T-shaped types are also to be found."

4) Sampson (1969) reported a unicellular archesporium in Hedycarya arborea. In contrast, Foreman (1984, p. 256) described that multiple archesporial cells were observed in Austrommathea elegans, Palmeria coriacea, and P. scandens.

5) The only report on this character is probably by Sampson (1969), who reported that megaspore tetrads are linear or less commonly T-shaped in Hedycarya arborea.

6) Corner (1976, p. 197) provided a description for the seed of Steganthera sp.: "Chalaza ? extended as in Hortonia, and perichalazal." According to Endress and Igersheim (1997, p. 137), the ovule of Tambourissa sieberi is "slightly perichalazal."

7) Based on observations of seeds of Kibara coriacea and Steganthera sp., Corner (1976, p. 194) stated that "the monimiaceous seeds were endotestal with tracheoidal cells."

**Table 1.** Taxa studied of Hortonioideae and Monimioideae (Monimiaceae), with their collection data and developmental stages.

Taxon	Subfamily	Collections	Developmental stages
<u>Monimia amplexicaulis</u> Lorence	Monimioideae	Mauritius. <u>H. Adersen 5196</u> (MO)	All stages
<u>M. ovalifolia</u> Thours		Mauritius. <u>H. Adersen 5303</u> (MO)	All stages
<u>M. rotundifolia</u> Thours		Mauritius. <u>H. Adersen 5172</u> (MO)	All stages
<u>Palmeria scandens</u> F. Muell.		New Caledonia. <u>B. R. Jackes</u> , no voucher	Very young to mature seeds
<u>Peumus boldus</u> Molina	Hortonioideae	Chile. <u>Valdavia</u> . X Region de los Lagos.	All stages
		<u>M. Riveros</u> , no voucher	
<u>Hortonia floribunda</u> Wight. ex		Sri Lanka. <u>P. Ashton 3786</u> (MO)	All stages

**Table 2.** Comparisons of embryological features in Monimiaceae.

\* New or revised data; \*\* data confirmed; NA, data not available. Features with neither single nor double asterisks are from literatures.

Characters	Monimioideae			Hortoniodeae	Mollinedioideae
	Monimia	Palmeria	Peumus	Hortonia	Hedycarva and other genera
<b>Anthers and microspores</b>					
Number of sporangia	Bisporangiate**	Tetrasporangiate	Tetrasporangiate*	Tetrasporangiate*	Tetrasporangiate
Anther wall formation	NA	NA	Dicotyledonous	NA	NA
Thickness of anther wall	6-7 cell-layered*	6-7 cell-layered	5-7 cell-layered**	NA	6-8 cell-layered
Anther epidermis	Flattened**	Flattened	Flattened**	Flattened**	Flattened
Endotecium	Fibrous**	Fibrous	Fibrous**	Fibrous**	Fibrous
Middle layers	Crushed*	Crushed	Crushed**	Crushed**	Crushed
Tapetum	Glandular*	Glandular	Glandular**	Glandular**	Glandular
Number of nuclei in a tapetal	2* <sup>1)</sup>	2	2**	2**	2
Cytokinesis in meiosis	Successive*	Successive	Successive**	Successive*	Successive
Predominant shape of	Isobilateral*	Isobilateral <sup>2)</sup>	Isobilateral*	Isobilateral*	Isobilateral or decussate <sup>3)</sup>
Number of cells in a mature	2-celled*	2-celled	2-celled**	2-celled**	2-celled
Anther dehiscence	By slit**	By slit	By slit*	By slit**	By slit
<b>Ovule, nucellus and megasporangium</b>					
Ovule orientation	Anatropous**	Anatropous**	Anatropous**	Anatropous**	Anatropous or hemianatropous
Number of archesporial cells	2-3*	NA <sup>3)</sup>	ca. 3*	NA	1 or multi-celled <sup>4)</sup>
Nature of nucellus	Crassinucellate**	Crassinucellate**	Crassinucellate**	Crassinucellate**	Crassinucellate
Thickness of parietal tissue	2-4 cells thick**	2-4 cells thick	3-4 cells thick**	2-4 cells thick*	3-4 cells thick
Shape of megasporangium tetrad	Isobilateral*	NA	Isobilateral*	Isobilateral*	Linear or T-shaped <sup>5)</sup>
Mode of embryo sac (e.s.)	Polygonum*	Polygonum	Polygonum*	Polygonum*	Polygonum
Shape of mature e.s.	Ellipsoid*	Ellipsoid**	Ellipsoid**	Oblong-ellipsoid**	Ellipsoid to obovoid*
Multiple e.s.	Not formed**	Not formed**	Not formed**	Not formed**	No
Antipodal cells	Ephemeral*	Ephemeral*	Ephemeral*	Ephemeral**	Ephemeral
Starchy grains in e.s.	Absent**	Absent*	Absent**	Absent	Absent
Nucellar cap	Formed**	Formed**	Formed**	Formed**	Formed
Nucellar beak	Not formed**	Not formed**	Not formed**	Not formed*	Not formed
Nucellar tissue in mature	Remains**	Remains**	Remains**	Remains**	Remains
Obturator	Not formed*	Not formed**	Not formed*	Not formed*	Not formed
Specialization of chalaza	Not specialized*	Not specialized *	Not specialized*	Not specialized*	Perichalazal <sup>6)</sup>
<b>Integuments</b>					
Number of integuments	2**	2**	2**	2**	2
Thickness of ii (early stage)	2-3 cells thick*	NA	2-3 cells thick**	2-3 cells thick**	2-3 cells thick
Thickness of ii (late stage)	3 cells thick**	3 cells thick**	2-3 cells thick**	3 cells thick**	2-5 cells thick
Thickness of oi (early stage)	3 cells thick*	NA	3 cells thick*	3-4 cells thick**	3-4 cells thick
Thickness of oi (late stage)	3-4 cells thick	3-5 cells thick**	3-4 cells thick**	4-5 cells thick**	3-5 cells thick
Histogenetic origin of ii	Dermal*	NA	Dermal*	Dermal*	Dermal
Histogenetic origin of oi	Subdermal*	NA	Subdermal*	Subdermal*	NA
Vascular bundles in ii (or	Absent*	Absent**	Absent*	Absent**	Absent

Table 2 Continued.

Characters	Monimioideae			Hortonioideae	Mollinedioideae
	Monimia	Palmeria	Peumus	Hortonia	Hedycarya and other genera
Vascular bundles in oi (or Micropyle formation)	Absent* By ii*	Absent** By ii and oi	Absent* Not formed**	Absent** By ii, occasionally not formed**	Absent By ii or not formed
Endothelium	Not formed**	Not formed**	Not formed**	Not formed**	Not formed*
Fertilization, endosperm, and embryo					
Path of pollen tube	Porogamous*	NA	Porogamous**	Porogamous*	Porogamous
Mode of endosperm formation	<u>ab initio</u> Cellular*	<u>ab initio</u> Cellular*	<u>ab initio</u> Cellular**	<u>ab initio</u> Cellular*	NA
Type of embryogeny	Onagrad	NA	Onagrad	NA	NA
Suspensor	Short*	NA	Short**	Short*	NA
Young seed					
Hypostase	Differentiated**	Differentiated**	Formed**	Formed**	Formed
Ramification of raphe bundle	Occurs**	Occurs**	Occurs**	Occurs**	Occurs
Testa multiplicative?	No*	No**	No*	No**	No
Tegmen multiplicative?	No*	No**	No*	No**	No
Thickness of testa	3 cells thick*	3-5 cells thick*	3-4 cells thick*	4-5 cells thick**	4-5 cells thick
Mature seed					
Aril or arilloid	Absent**	Absent**	Absent**	Absent**	Absent
Endosperm in mature seed	Present**	Present, oily**	Present**	Present**	Present
Perisperm	Absent**	Absent**	Absent**	Absent**	NA
Embryo curvature	Straight**	Straight**	Straight**	Straight**	Absent
Cotyledons	Straight**	Straight**	Straight**	Straight**	Straight
Rumination	Not ruminated*	Not ruminated**	Not ruminated*	Not ruminated**	Not ruminated*
Mature seed coat					
Seed coat type	Mesotestal-	Mesotestal-	Mesotestal-	Mesotestal-	Endotestal <sup>7)</sup>
Thickness of testa	3 cells thick*	3-5 cells thick*	3-4 cells thick*	4-5 cells thick**	4-6 cells thick
Features of exotestal cells	Membranous, thin-walled.	Tanniniferous, thin-walled and	Tanniniferous*	Tanniniferous**	Lignified or tanniniferous
Thickness of mesotesta	1 cell thick	1-3 cells thick	1-2 cells thick	2-3 cells thick	2-3 cells thick
Features of mesotestal cells	Tracheoidal*	Tracheoidal*	Tracheoidal*	Tracheoidal*	Thin-walled or slightly thick-walled
Thickness of endotesta	1 cell thick	1 cell thick	1 cell thick	1 cell thick	1 cell thick
Features of endotestal cells	Tracheoidal*	Tracheoidal*	Tracheoidal*	Tracheoidal**	Tracheoidal <sup>7)</sup>
Thickness of tegmen	0*	0**	0*	0**	0 or 4 cell(s) thick
References	Present study; Endress and Igersheim (1997); Lorence (1985)	Present study; Corner (1976); Endress and Igersheim (1997); Foreman (1984)	Present study; Endress and Igersheim (1997); Kamelina (1981); Mauritzon (1935)	Present study; Corner (1976); Endress (1980); Endress and Igersheim (1997)	Corner (1976); Endress and Igersheim (1997); Foreman (1984); Lorence (1985, 1987); Sampson (1969, 1977); Vyshenskaya (1988)



